Dissertation
submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany
for the degree of
Doctor of Natural Sciences

presented by

Diplom- Daria Gavriouchkina
born in Moscow, USSR
Oral-examination: .....

The *Platynereis* Cell: from morphology to single-cell amplification

Referees: Dr. Anne Ephrussi
Prof. Dr. Thomas Holstein

Summary

All organisms are composed of cells. These cells display a plethora of different morphologies and functions. Since their discovery in their in the seventeenth century their secrets and mysteries have fascinated philosophers and natural scientists alike. In the first part of this thesis I describe experiments performed on a very curious organism; the zebrafish, Danio rerio, whose every cell can carry out a function relegated to only a few chosen cells in other organisms - that of light perception. In order to investigate this phenomenon a genome-wide screen for light-induced genes and miRNAs was performed in the zebrafish embryo, at a stage when photoreceptive organs or structures were not present yet . We revealed the regulatory mechanism that controls these processes. Using a computational approach in combination with knock down and over expression studies we demonstrate that the PAR bZip transcription factor TEF α plays a key role in the regulation of the majority of light-induced genes during early zebrafish development. Since we show that tef alphaα transcription is under circadian clock control, our data suggest that fish embryos anticipate the daily exposure to radiation and the ensuing damage.

This capacity to respond to light directly is common to all zebrafish cells but is restricted to only a chosen few in many organisms, in which specific cells called photoreceptors are required for either circadian or non-circadian light perception. Cells categorized as a function of their function, morphology or gene expression are called cell types. Considerable interest has been taken in the evolutionary conservation of cell types between different phyla.

In the second part of this thesis, I present work performed in the lab of Dr. Detlev Arendt on the marine polychaete worm Platynereis dumerilii, performing exploratory method development to dissociate larval and adult worm into individual cells in the most efficient fashion, followed by characterization of the resulting single cell suspensions using morphological and molecular methods. Attempts at cell culture from Platynereis dumerilii larval and adult cells as well as other marine organisms are described, demonstrating that primary cell culture can be achieved in these organisms but that further effort is necessary to identify appropriate conditions necessary for the proliferation and survival of these cells.

Zusammenfassung

Alle Organismen sind aus Zellen aufgebaut, die seit ihrer Entdeckung im 17. Jahrhundert Philosophen und Naturwissenschaftler gleichermassen faszinieren. Zellen zeigen eine Fülle unterschiedlicher Morphologien und Funktionen. Im ersten Teil dieser Arbeit geht es um die Wahrnehmung von Licht im Zebrafisch, bei dem im Gegensatz zu anderen Tieren jede Zelle lichtempfindlich ist. Um dieses Phänomen zu untersuchen, habe ich ein genomweites Screening nach lichtinduzierten Genen und MikroRNAs im Zebrafischembryo durchgeführt, in einem Stadium, in dem noch keine Augen oder andere lichtempfindlichen Organe entwickelt sind. Mit diesen Experimenten konnten wir die der Lichtinduktion zugrundeliegenden genregulatorischen Prozesse beschreiben. Mit Hilfe von bioinformatischer Analyse in Verbindung mit dem experimentellen Ausschalten und der Überexpression von Genen konnten wir nachweisen, dass ein bestimmter Transkriptionsfaktor der PAR bZip Familie, TEF alpha, eine wichtige Rolle bei der Regulation lichtinduzierter Gene im frühen Zebrafischembryo spielt. Da die Transkription von TEF alpha circadianer Kontrolle unterliegt, legen unsere Daten nahe, dass die Fischembryonen die tägliche Strahlungsexposition und damit verbundene Schädigung der Zellen vorwegnehmen und entsprechende Gene vorsorglich aktivieren.

Bei den meisten Tieren ist Lichtempfindlichkeit vor allem in spezialisierten Lichtsinneszellen ausgeprgt. Solche aufgrund ihrer Morphologie, Funktion und Genexpression kategorisierten Zellen bilden einen ”Zelltyp”. Die mgliche Homologie von Zelltypen zwischen verschiedenen Tierstmmen hat in jüngster Zeit grosses Interesse erregt. Im zweiten Teil meiner Arbeit beschreibe ich Experimente, die ich im Labor von Dr. Detlev Arendt am Meeresringelwurm durchgefhrt habe. Ich habe versucht, Methoden zur effizienten Dissoziation von embryonalen, larvalen und juvenilen Würmern in Einzelzellen zu entwickeln und im Anschluss daran die daraus resultierende Zellsuspension morphologisch und molekular zu charakterisieren. Ansätze zu einer Etablierung einer Zellkultur von larvalen und adulten Meeresringelwrmern sowie anderer Meeresorganismen werden ebenso vorgestellt, welche die Machbarkeit einer solchen Kultur zeigen. Weitere Anstrengungen werden vonnöten sein, um genauere Wachstums- und Überlebensbedingungen für diese Zellen in der Kultur herauszuarbeiten.

Acknowledgements

I would like to thank Pr. Dr. Detlev Arendt for giving me the opportunity to work in his lab and for taking over the burden of being my PhD advisor from Dr. Carl Neumann, and introducing me to the intricacies and challenges of "evo-devo", a young and exciting field.

From my time under the supervision of Dr. Carl Neumann, very special thanks are due to Dr. Marcus Dekens. His direction, assistance and guidance have been invaluable on the zebrafish project, having taught me far more than only techniques and scientific reasoning. He has been much more than a supervisor and a mentor, having become a true friend and a rare inspiration in terms of what true passion for science looks like. I would also like to acknowledge the members of the Neumann lab: Dr. Marlene Rau, Dr. Johan Ledin, Dr. Veronica Gonzalez-Nunez, Mrs. Sabina Radeva, Ms. Valentina Nocco, Dr. Sergey Prykhozhyj, Mrs. Sabine Fischer and Mr. Dan Edvardsson for expert fish care.

My transition from the Neumann lab was made possible by my TAC, Dr. Lars Steinmetz and Dr. Anne Ephrussi. I would like to particularly thank her for her intervention in difficult situations and organizing the change of lab during a difficult time for me and for accepting to be a member of my defense committee.

I would furthermore also like to thank all the present and former members of the Arendt lab: Mr. Benjamin Backfisch, Dr. Elia Benito-Gutierrez, Dr. Foteini Christodoulou, Dr. Antje Fischer, Dr. Mette Handberg-Thorsager, Dr. Peter Hantz, Ms. Nicola Kegel, Dr. Tomas Larsson, Ms. Antonella Lauri, Dr. Heather Marlow, Ms. Silvia Rohr, Mr. Oleg Simakov, Ms. Heidi Snyman, Dr. Raju Tomer, Ms. Maria Antonietta Tosches, Dr. Ferdinand Marletaz for support and Ms. Diana Bryant for expert worm care. Ms. Antonella Lauri deserves special thanks for the original impetus that started the cell culture project and would not have taken place without her enthusiasm. I am particularly grateful to Dr. Ferdinand Marletaz for critical reading of this document and for his undwindling support and encouragement during these last very difficult months of this PhD, without which this thesis would not have been possible. The scientific discussions we have shared have taught me a great deal about far more than I could have imagined about evolutionary theory.

I would also like to express my thanks to Tomi Ivacevic, Jens Stolte for their help on the microarray and microRNA qPCR experiments and Dr. Vladimir Benes for support and expert advice throughout my time at EMBL on all the projects that I was involved in. I could not forget all the members of the mechanical workshop for creative solutions to the mind boggling dilemmas posed by circadian setups. Dr. Nelly Odintsova (Insti-

tute of Marine Biology FEB RAS, Vladivostok, Russia) provided ideas and support for cell culture experiments.

Last but most certainly not least, I offer my regards and blessings to all of those who supported me throughout my time at EMBL, Valerie Hilgers for her "no nonsense" advice and kindness, Michal Karzynski who opened my eyes to far more things that he will ever suspect, Priyanka Saksena, Raga Krishnakumar, Lu Han and Joanna Howard for our enduring friendship, and last but most certainly not least my parents, who have always supported me.

Contents

Introduction

For a considerable portion of mankind's history, the classification of life has been limited to naming purposes and to rather practical considerations, best popularized by the infamous disagreement between the scientist and the sailor, in Jules Verne's phantasmagoric "Vingt mille lieux sous les mers", in which Ned Land classically classified living beings into those that could be eaten and those that could not. This is indeed most probably the most important and useful definition for the subsistence of humanity. However, once toxicity and flavor is set aside, one can concentrate on the more philosophical aspects of the diversity of life on Earth and therefore on their origin(s). Indeed, since the mid 19th century thanks to the groundbreaking works of Lyell, Lamarck, Malthus and Wallace, and the much cited 1853 *magnum opus* of Charles Darwin "The origin of species", the concept of descent has increasingly become a subject of debate. One of the key questions was: "which characters are ancestral and which evolved secondarily". Geoffroy Saint-Hilaire already in the 18th century argued that a shared ancestral organization indicated a common descent, whereas his counterpart Cuvier on the other hand thought that only functional constraints shaped the characters of living beings. Out of these controversies and many others, stemmed the field of comparative biology, whose goal was to apprehend properties of organisms in an evolutionary context. Another key figure in this field was Willi Hennig, who in the 1950ies, established the cladistic approach, which provided for the first time a rigorous comparative classification method. These century-old ideas, had to go to through a sudden and sharp reappraisal during the past 30 years due to the undeniable influence of molecular biology on classical comparative biology. The surprising conservation of genomic organization and of the molecular processes underlying development and animal physiology, raised further questions on the origins and mechanisms that brought about the diversity of life on Earth [1].

Invariably the debate comes down to the concept of homology which we will first discuss. Subsequently we will cover the ideas that have stemmed from the latter concerning the different proposed natures of a putative ancestor. Some authors have proposed that this argument could be better understood in terms of the evolution of cell types, as cell type number can be considered as one of the best descriptor of organismal complexity . We will therefore, then go on to discuss the notion of cell type, its limitations and possible contributions to the evolutionary debate.

Evolution of Animal Complexity: Unanswered Questions

The notion of homology

The entire debate of evolution of animal forms finally lies on what might at first appear as a very simple notion - that of homology, or that two structures are similar due to descent from a common ancestor that possessed the trait. Unfortunately, like most things in life, application of this principle of homology is far from simple.

Lecointre distinguished between primary homology also known as structural homology (*homologie structurelle*) and cladistic homology or homology by filiation (*homologie par filiation*). Structural homology is based on the connectivity principle put forth by Geoffroy Saint Hilaire and later reformulated by Owen in 1843. Two structures are considered homologous in this instance if they are connected to similar neighboring structures in the same topological fashion, carrying out similar functions and possessing similar morphologies. For example, whether the human radius or that of dolphins or bats, are in contact with another bone - the ulna in a distal position to the carp bone, proximally to the humerus. This is observed irrespective of the shape of the bone or the function. Whether it is flat as in the dolphin or elongated as in the bat and whether it is used for swimming or flaying , the structures are considered homologous due to their environment. Thus, homology is inferred in a stepwise manner. Based on the connections observed from a structure. The fifth metatarsal whether that of a turtle or of a human is always located in the same position relative to other bones. However, in the sphenodon and the lizard, it is tilted, whereas in the turtle it is not. Therefore, using the principle of parsimony one would assume that the lizard and the sphenodon had a common node, indicating that the tilted fifth metatarsal appeared in a hypothetical common ancestor of sphenodons and lizards, that was not shared by the human and turtle. Based on this tree the second type of homology - cladistic homology is inferred.

However, the situation is not so simple. A third kind of homology that does not fit into either of the definitions of homology described above has been noted by numerous authors, refering to cases where ambiguity exists about the exact character state in the common ancestor. In such situations, similar characters could be observed in several but not all branches of the same clade [2], allowing certain phenotypes hypothetically possessed by a common ancestor to to be lost, gained again and modified. As a result some similarities are considered homologies within the scope of the loose definition and homoplasic within the scope of the cladistic definition (reviewed in [2]). West-Eberhardt dubbed this third kind of homology *broad-sense homology* in which similarity is not due to unbroken descent from an immediate common ancestor as it is the case for cladistic homology [3, 2]. Alternatively, Stephen J. Gould also discussed extensively possible occurrences of parallel evolution that he called *grey homology* [4], whereas Shubin referred to molecular processes underlying such processes with the the term *deep homology* [5].

Although, these concepts were originally developed to refer to organs and body parts, and only later were applied to molecular biology, the latter does indeed allow the assignment of different elements of complex traits to their underlying building blocks. A classic example of this mixed broad-sense homology applied at the molecular level is the eye. The development of both the *Drosophila* compound eye and the vertebrate

eye rely on the expression of the Pax6 gene, although, the two eye structures have been determined to have evolved independently [6]. At a broader scale, the insect hormone ecdysone has been observed in plants [7]. Opposingly, West-Eberhard has furthermore pointed out that distinct developmental pathway can lead to similar conserved phenotypes [2], such as the multiple methods employed by different neotenous salamanders for preventing metamorphosis [8, 6], demonstrating a complex form of end-point convergence. The neural tube although it is considered a phylotypic and archetypical trait among chordates, is not formed in the same way in all chordates. In teleost fish and lampreys neurulation takes place by a coalescence of cells as a solid rod that subsequently becomes hollow, whereas in all other chordates, this method is used only in the tail. The head and trunk neural tube is created by the folding of the anterior ectoderm [2].

Some authors have recently suggested that these observed cases of 'broad-sense' homology or parallel evolution may be due to the repeated involvement of conserved genetic networks in the making of convergent organs [5]. Shubin, Tabin and Carroll go even further in claiming that: " new structures need not arise from scratch, genetically speaking, but can evolve by deploying regulatory circuits that were first established in early animals". Nevertheless, the quite subtle theoretical issue has a massive impact on the way we see the ancestral of bilaterian animals. Indeed, the overall interpretation of the growing amount of molecular evidence collected in many different lineages heavily rely on our ability to employ them to infer ancestral traits.

Homology at Molecular Level

If homology reasoning can be distinguished no longer purely based on morphological and developmental characters, but based on the conservation of expression of transcripts and proteins, shouldn't the rules governing the concepts of homology as applied to organisms also apply to the molecules themselves as well? At this point, can one speak of true conservation of molecular markers without taking into account their function? Classically, a primary level of genes homology can be inferred from phylogenetic analysis of their molecular sequences, giving the opportunity to trace back the origin of multigenic families by distinguishing between paralogs arisen through lineage specific duplication and orthologs that retained their vertical descent. However, at a broader scale, careful examination of functional data points out that the ancestral functions of gene or gene families cannot necessarily be inferred from sequence information alone.

For example, the function of transcription factors is to induce the transcription of a set of target genes by binding to select DNA sequences in their promoters and enhancers. The case of Pax6 gene remarkably illustrates this dilemma. The Pax6 gene has long been lauded as a universal 'master' control gene expressed in the development of a variety of morphologically distinct eyes ranging from *Drosophila* to vertebrates [9, 10, 11]. The *Drosophila* Pax6 homolog eyeless mutants lack eyes , whilst eyeless or twin of eyeless overexpression results in ecotypic eyes [12, 13]. Similarly, in mice Pax6 mutants suffer from complete or partial absence of the iris in the heterozygous state and homozygous mutants possess small eyes or lack eyes altogether [14]. Mammalian Pax6 has been demonstrated to activate rhodopsin and crystallin gene expression [15, 16, 17, 18]. A second family of Pax genes - the Pax2/5/8 subfamily comprises

a single member in Drosophila and three members (Pax2, Pax5, and Pax8) in mammals. These genes have been implicated in the development of ommatidial cone and pigment cells , mechanosensory bristles in *Drosophila* and kidney, eye, inner ear defects, midbrain, cerebellum, and B-lymphopoiesis in mammals [19, 20, 21, 22, 21, 23, 24]. Kozmik and colleagues demonstrated that the cnidarian PaxB gene corresponds to an ancestral Pax gene, and is a functional hybrid of Pax2/5/8 and Pax6, based on sequence similarity and DNA binding specificity. The cnidarian PaxB gene could rescue the *Drosophila* Pax2 mutant was able to induce ectopic eyes in Drosophila. In cnidarians, PaxB is responsible for the activation of crystallin genes in a manner resembling the Pax2/5/8 rather than the canonical Pax6s [19]. Kozmik and colleagues showed that the ancestral gene PaxB was responsible for eye development in cnidarians and that Pax6 arose from a common ancestor with PaxB in higher metazoans only after the separation of Cnidaria from Bilateria. Hence, Pax6 evolved by duplication from an ancestral PaxB-like gene only after the separation of Bilateria from Cnidaria, indicating the independent origin of eyes in cnidarians and higher metazoans [19].

This example demonstrates that pure sequence similarity is not sufficient to ascertain the ancestral function of genes, such as the activation of conserved targets for a transcription factor. If we return to the example of transcription factors, a conserved DNA-binding domain is considered to be proof of conservation of function which questions the conservation of target cis-regulatory sequence. However, recently, evidence has accumulated demonstrating that the non-coding stretches of DNA are not conserved. Accumulating cross-species comparisons, demonstrate that although the structure of a small number of transcription factors is conserved their respective target motifs are not. Dermitzakis and Clark observed extensive divergence within the nucleotide sequence of transcription factor binding sites between primates and rodents with up to 40% of the human functional sites not functional in rodents [25]. Using chromatin immunoprecipitation and microarray analysis (Chip-Chip) on three species of *Saccharomyces*, Borneman and colleagues demonstrated that transcription factor binding sites had diverged faster than ortholog content [26]. Similarly, Chip-Seq experiments in five vertebrate livers demonstrated that each transcription factor displayed highly conserved preference in terms of DNA target motifs, yet that most events of binding are specific to each species and rarely match stretches conserved in all five species [27]. Authors attributed this discrepancy to modifications of bound target motifs in each lineage. Hence, one can no longer assume that high sequence homology and DNA binding domain conservation is sufficient to infer homology of function, particularly at the regulatory level.

Homology at the Organismal Level: Conflicting Views of Urbilateria

The goal of defining homology is of course to infer information about extinct ancestral species and the way in which the currently observed variability of life on Earth came about. Different schools of thought have proposed contradictory models as to the complexity of respective ancestor hypotheses. New light was shed into this only in the mid-1970s with the publication of Stephen Jay Gould's "Ontogeny and Phylogeny" and the first papers that started the field of a new field dubbed "evo-devo" [28]. The comparison of the molecular mechanisms utilized at early developmental stages to set

up the respective bauplans of different organisms surprisingly demonstrated the underlying commonality of development at the molecular level [1].

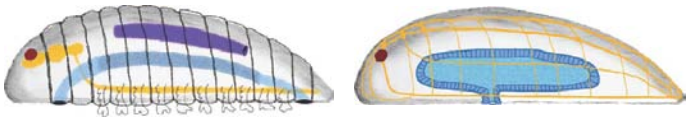
In his magnum opus "The Structure Of Evolutionary Theory", Stephen Jay Gould described two warring parties in the century-old debate aimed at determining the nature of the bilaterian ancestor aka 'Urbilateria' [4]. The European school descend from the "transcendant morphologists" movement on one hand, related to the German school of thought headed by the great German romantic philosopher Goethe [29, 30, 31]. This school of thought argues for a complex ancestor endowed with many of the traits observed in modern animals, rather than attributing them to independent convergent evolution.

Authors party to this view feel that there is strong evidence among model and non-model system of for conservation of a number of developmental pathways involved in anterior-posterior patterning [32], eye development [9], heart morphogenesis [33], immune system [34] and body segmentation [35, 36]. The views put forth by this school were further expanded when studies were extended to a further understudied clade - the Lophotrochozoa, notably with the example of the annelid worm *Platynereis dumerilii* [37, 31]. The complex version of the bilaterian ancestor would have possessed a metameric body plan, with gut, mouth, anus, heart, eyes and central nervous system containing mushroom body-related structure [31].

Conversely, an Anglo-American school of thought on the other hand argues for a very simple ancestor, similar in many ways to an acoel, and allows for much convergent evolution among traits found in modern animals [38] (See Fig. 1). The proponents of this view argue the assumption of a complex ancestor would imply a high frequency of independent loss of these complex organ systems, not found in a number of modern organisms, assuming appropriate phylogenetic sampling is used [39]. Some may argue that an independent loss would hypothetically occur more 'easily' than an independent convergent gain in evolution, however although this notion appears quite intuitive little data is available to substantiate it. Furthermore, they argue that the gene expression studies underlying their opponents views are biased, proposing instead that a highly conserved "toolkit" of genes exists in virtually all metazoans and that their incorporation into interacting networks responsible for the formation of complex tissues and organ systems may have been ancestral rather than the existence of the complex structures themselves [38]. There are also of course numerous compromising views situated on a gradient between these extreme positions between these warring factions whose precursors have existed since the mid-XVIIIth century.

When Smaller May Be Better

The debate is still open and will most probably remain so for many years to come. Possible directions of research that could settle the debate could include a increased taxonomic sampling, notably in the understudied Lophotrochozoan clade. A more extensive taxonomic sampling would not only simplify distinguishing derived characters from ancestral ones, but can lead to surprising observations. A recent investigation of hemichordate anterior-posterior neural patterning, for example, showed that vertebrate-like patterning, which was previously considered specific to vertebrates alone, due to lack of similarities with *Drosophila*, may indeed be more ancestral, and to have existed



(*c*)

character	genes	complex	simple
AP patterning	*HOX*		
DV patterning	*TGF*b/*BMP* 2/4 *sog*/*dpp*		
posterior patterning	*evx*, *cdx*		
central nervous system	*otx*, *emx*, *six*3/6, *HOX1*		
photoreception 'eyes'	*PAX6*, *RX*, *opsin*		
heart	*tinman*		
segmentation, segmentation clock	*hairy*, *engrailed*, *notch*/*delta*		
regionalized through gut	*HNF*b 3, *GATA* factor *goosecoid*, *brachyury*		
appendages	*Distal-less*/*DLX*		

Figure 1: Comparison of complex and simple hypothetical ancestor of bilaterians. Top left panel: the complex depiction of the stem species of the Bilateria . Top right panel: the ground pattern of the Bilaterian. Bottom panel: comparison of the gene expression data and organs present in the hypothetical ancestor under either model. Figure modified and reproduced from [38].

in the ancestor to vertebrates and hemichordates [40]. Moreover, a more exhaustive gene repertoire could be used in the the comparison. Extending gene expression studies outside the canonical gene regulatory networks and conserved molecular toolkit, by determining gene expression profiles for full transcriptomes would make comparative studies amenable to statistical analyses and eliminate the much criticized bias in the gene analysis. Such a high throughput approach is becoming increasingly affordable with the genome-sequencing technology previously unavailable to non-canonical model organisms.

Yet, another possible line of thinking to consider this paradox outside the standard whole organismal and organ- or organ-biased scale and instead to focus down to the cellular level common to all living forms and investigate gene expression regulation within individual cells. Sadly, tumorogenesis reminds us of how the developmental and cell differentiation programs can escape during the course of individual life, and that individual cell proliferation can take over tight genome regulation [41]. Gene profiling at the cellular level has already been attempted in the evo-devo field. Tomer and colleagues have taken advantage of cell-level resolution of confocal microscopy to profile gene expression profiles from wholemount *in situ* hybridization [31]. Using nuclear and neuronal scaffolding staining the authors have successfully mapped individual *in situ* patterns back to images of whole larvae at a cellular level. This method could be a powerful tool if implemented to a larger set of taxomomic groups allowing cellular-level interspecies comparisons.

Therefore, in the following section we will discuss the interest of cellular-level studies in an evolutionary context and the validity of the 'cell type' concept , its advantages and limitations in investigations in this regard.

A Primer To Cell Types

The Advent of Cellular Biology

Indeed, one of the few canons of biological theory which was established far before evolutionary theory and remains key notion to this day, is that of the fundamental building block of life - the cell. When the 17th century English natural philosopher Robert Hooke observed a cork using one of history's first microscopes, he noted:

> I no sooner discern'd these (which were indeed the first microscopical pores I ever saw, and perhaps, that were ever seen, for I had not met with any Writer or Person, that had made any mention of them before this) but me thought I had with the discovery of them, presently hinted to me the true and intelligible reason of all the Phenomena of Cork [42].

Hooke's quixotically phrased exclamation may have seemed boorish and arrogant to his contemporaries, yet today it is the principal aspect of "cell theory", which gained recognition only after the work of Theodor Schwann, Matthias Schleiden ("all living beings are made of cells"), and Rudolf Virchow ("*Omnis cellula e cellula*") over two hundred years after Hooke pronounced these infamous words. These groundbreaking

works set the scene for the overwhelming field of cytology and histology, which characterized a plethora of surprising and exotic cells from a variety of animal or vegetal forms and stages (Fig. 2). Ironically, the wide variety of cells observed also posed an unexpected dilemma in defining the very notion of a cell. Prokaryotic cells of bacteria and archaea do not possess a nucleus, yet are endowed with a number of traits absent in eukaryotes. Although, one could assume that when speaking of a population of unicellular organisms, it is no longer a question of different cell types, but of different organisms, such a view could be misleading. Among some photosynthetic bacteria (*Nostoc*), heterocysts can occur at certain times. They are a"differentiated" form of nitrogen-fixing bacterial cell within a filament of bacteria that is specifically isolated from nitrogenase inhibiting oxygen [43]. Whereas among eukaryotes, a diversity of cells can be observed both in unicellular and multicellular organisms. When an acceptable definition of a cell could finally be agreed on, it were described as "small membrane-bound compartment filled with a concentrated aqueous solution of chemicals that form living organisms" [44]. The lack of precision in this definition may seem shocking at first. As cells are at the very center of our understanding of the richness of life, it would be an anathema to admit that we do not really know what they are. An artificial membrane bound pocket filled with a solution of salt inserted in a living being would then constitute a cell? This kind of *ad extremum* reasoning can only lead to absurd argumentation. Instead, if one were to look at this definition a little deeper the necessity to utilize such indefiniteness indeed becomes clear - the vast diversity of cells in existence is so broad that it impedes such an attempt. After all cells have been described possessing a single nucleus, multiple nuclei forming a syncytium as for example in the much studied early *Drosophila* embryo, without a nucleus as in bacteria and without DNA altogether as for example in mammalian erythrocytes.

To Be or Not To Be A Cell Type

The lack of precision in defining a cell also indicates a deeper difficulty not only in finding a common uniting principle between the vast richness of cell characteristics observed, but also poses a challenge to organize and classify these cell types in a logical and coherent manner. Although Kornblum and Geschwind have deemed this challenge *the fundamental question* in fields such as stem cell biology [45], the nature of this difficulty stands out as obvious when one attempts to find a definition of a cell type. Standard definitions encountered in textbooks and reviews emphasize two classical criteria: common function and morphology [46, 47, 48, 49, 50].

Although, quite intuitive, these criteria could be a quite misleading. When one speaks of cell morphology, there is of course the question of the level of resolution of the instruments used to evaluate such morphology. Cell type morphology observed via light microscopy is limited to the observation of subcellular organelles at a resolution of 0.5 microns (Fig. 2 (A-B)). Confocal microscopy has a resolution of 160-180 nanometers (Fig. 2(D-E)). Using advanced staining and microscopy methods such as STORM or PALM and TIRF, imaging resolution down to the lower nanometer range has been obtained [51, 52, 53]. Conversely, electron microscopy allows the observation of organelles a hundred times smaller (Fig. 2(F-G)), revealing cytoarchitectural features that define cell morphotypes [44]. Electron microscopy has played an impor-

tant role in settling debates concerning flatworm phylogeny based on theories concerning the commonality tissues putatively syncytial tissues which turned out to be cellular [54, 55].

The ”common function” argument raised above is also important, however it also raises more questions that it settles. Not only is the presumption of single function per cell dangerously misleading, but the functions of all cells are not yet known [56]. Conversely, Schwann cells and oligodendroglia share functions yet are acknowledged as distinct cell types [45].

When comparing the neural crest cells between different organisms Raff proposed the following criteria in order to determine whether two cells belonged to the same cell type:

1. cell fate
2. morphology and behavior
3. time and place of origin
4. cell lineage-restricted gene expression
5. cell lineage

Although at first sight these criteria may appear overly restrictive, closer reflection yields only further questioning. The term cell fate is intrinsically linked with that of differentiation, which refers to the development of specialized cell types [62], and implies a common function. Committed cells can in certain conditions dedifferentiate into other cell types, whereas stem cells have been defined as self-renewing cell populations that can differentiate into multiple distinct cell types [63]. Numerous organisms possess a considerable amounts of putative stem cells allowing for a high cell plasticity to fit the needs of the organism [64]. Commonly, during the process of regeneration, cells have been shown to take up a new fate, modifying their expression profile, function and morphology [65, 66]). For this reason, one can distinguish between the developmental cell fate and the real cell fate, in which the developmental fate of a cell or region represents what the cell or region differentiates into during embryonic development [44, 67, 68].

Within different tissues, differentiated cell types have been observed to develop from a variety of progenitor cell lines. Cell types distinguished only within the context of the tissues or organs in which they reside despite the fact that numerous cell types can be found in different tissue types and organs. Alberts and colleagues distinguished 210 functionally distinct human cell types in 20 cell classes, whereas Vickaryous and Hall spoke of 411 cell types [69]. Caution should be taken in making comparisons, as some morphological and functional properties of these cells may be homologous, but do not maintain a ”one-to-one relationship with each other. Thus, cell lineage may imply the descent from a common progenitor that can indicate a common origin” [70].

Finally, cell lineage-specific gene expression is a very tempting criterion. This approach has been particularly fruitful for disentangling blurry gene-to-phenotype relations [71]. For example, Striedter and Northcutt stressed that the archenterons of lampreys, sharks, teleost fish, salamanders, frogs and lungfishes while all homologous

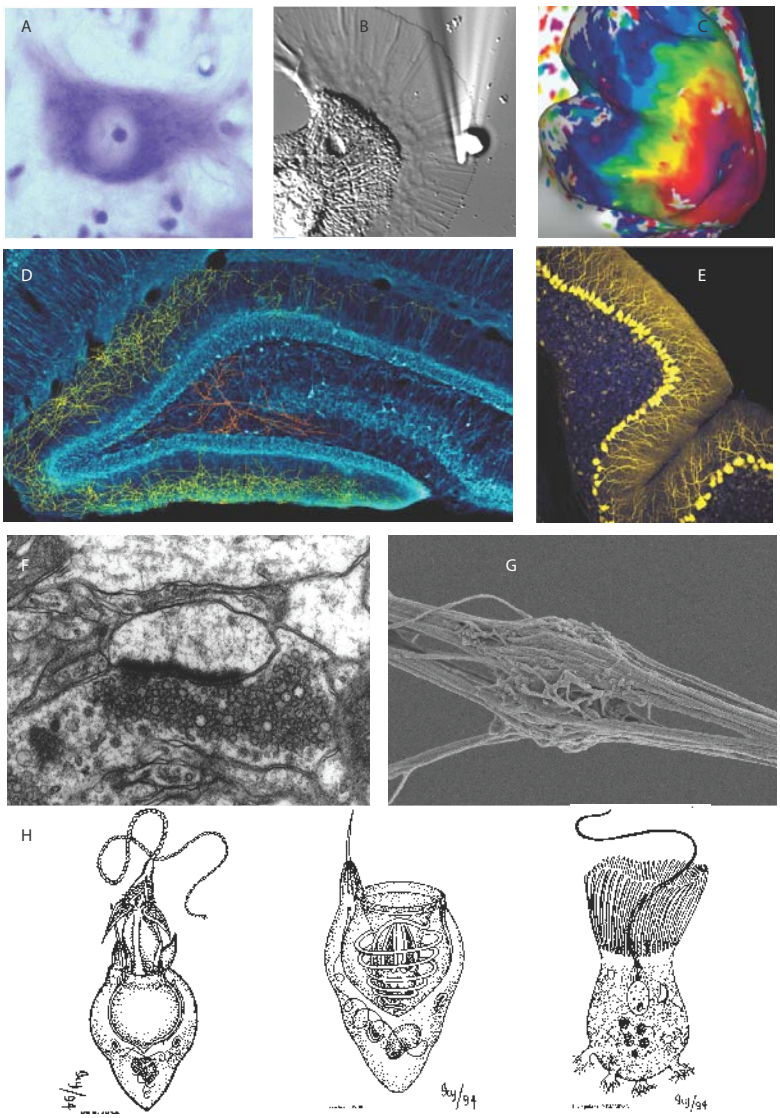


Figure 2: (A) A neuron stained with Nissl staining, observed using light microscopy [57]. (B) An axonal growth cone observed using light microscopy [58]. (C) MRI on visual cortex of living human subject [59]. (D) A single neuron's soma and dendrites (at center, orange) and the dense branches of its axon (yellow), in rodent hippocampus [59]. (E) Purkinje cells expressing fluorescent reporter gene in cerebellum observed with confocal microscopy [59]. (F) Transmission electron micrograph of a synapse [60]. (G) Scanning electron microscopy image of neurons in culture [59]. (H-J) Cell types of particular interest synapomorphies of Poriferans and Cnidarians (H from left to right: Cnidocyte in two different views and Choanocyte at far right) [61]

as embryonic guts make use different non-homologous genes in their developmental pathways [70]. At present, more and more studies isolate cell populations based on the expression of fluorescent reporter genes or antibody staining using methods such as FACS. This kind of approach may mask an underlying heterogeneity, as a given cell population may indeed appear homogenous in its expression of one given marker, yet display surprising heterogeneity in terms of other markers. Cell classification based on full transcriptome mapping may appear as the ultimate method for cell characterization. Stuart Kauffman derisively mused that:

> A cell type is a particular pattern of gene expression; different cell types are different patterns of gene expression; by and large the genome in all cell types in an organism is identical [...] As we all know, a higher eukaryote has on the order of, say, 20,000 to 100,000 structural genes and an unknown number of regulatory gene. Focus only on the structural genes, simplify and conceive of each gene as simply active or inactive. Then the potential combinations of gene activity are at least $2^{20000} = 10^{6000}$, a number vaster than the number of hydrogen atoms which might fill the known universe. We would not want to say that any such pattern is a cell type, I suppose. But why not? And what is a better idea?

However, single cell gene expression assays are increasingly demonstrating that transcription does not proceed in a continuous manner. Instead there are bursts of transcription. So the question of cell type specific gene expression should only be considered in a temporal manner. Moreover, studies investigating such events are increasingly demonstrating that cell-to-cell variability has been severely underestimated (see Chapter 3), raising only further questions on the definition of a cell type [72, 73, 74, 75, 76, 77].

The Cell Type: An Operational Concept?

An alternative view could also be proposed. Alberts' definition of a cell is vague, because the concept of a cell type is inherently an operational concept, a simplification valid only within the framework of a given theory or model, which itself is also a simplification of the complexity of a biological system. It is thus important to take care that a "cell type" is not an absolute property and cannot be utilized outside the context of the model they were put forth in, as the same cell viewed from the perspective of different paradigms may fall into several sometimes contradictory classifications. In 1964, Leblond, for example, classified cells based on their proliferative behavior into three groups: static cells (for example neurons), growing cells (transiently amplifying cells) and renewing cells (for example stomach epithelial cells). This classification was based on two criteria - the presence or absence of mitoses and the duration of the cell cycle of descendant cells relative to that of the host cell [78]. A neurobiologist studying cell neuronal differentiation would find this definition quite unsatisfactory, as it would not correspond to the paradigms under study, by placing practically all neurons in the first group, whereas a cell cycle researcher may be quite content with it.

Therefore, if a researcher defines his or her cell type within the constraints of the

system studied, any study utilizing different tools could putatively generate a terminology specific to the tools and field studied with its inherent advantages and constraints.

Cellular approaches to metazoan evolution

It is perhaps because of the difficulty in defining the cell type that so few studies have adopted cell-level approach in the evolutionary context. Some authors have taken advantage of cell types defined by others to carry out comparisons of cell morphotype numbers to evaluate the complexity of different groups of organisms. Others have characterized cell types based on criteria specific to their study. Some non-exhaustive examples of each will be discussed below.

Cell morphotype numbers applied to evolution

Cellular morphologists have classified cells based on electron and light microscopy images for many years. Some cell types display such a typical morphology that they have been lauded as phylum distinguishing synapomorphies, as for instance the Poriferan choanocyte and Cnidarian cnidocyte cells (Fig. 2(H)) [79]. These descriptions have been dubbed ”cell morphotypes”. Valentine and colleagues have taken advantage of these descriptions. Although the bias towards anthropocentrism can be easily felt and the how certain rare species have obtained cell type characterization remain dubious, these estimates provide an interesting opportunity to measure the complexity of different organisms. Table 1 (reproduced from [44]) lists the number of cell morphotypes reported in individuals belonging to each generally recognized metazoan phylum.

Valentine and colleagues plotted the maximum estimated cell type number for all metazoan species at different time points and demonstrated that there is an increase throughout the Phanaerozoic Era [80]. Authors postulated that if their common ancestor had only two cell types, the number of cell types could only increase. Cell type number decrease was also observed in Pogonophora, and in obligate parasitic phyla as Acantocephala, Rhombozoa, Orthonecta and In order to understand the differences and similarities, the homologies and homoplasia at key developmental stages of different animals the comparisons have to be carried out at a comparable scale.

Homology of Cell Types: Case Studies

Although, it is widely acknowledged that only genetic material and subcellular organelles such as mitochondria or centrosome are subject to inheritance and therefore selective pressure, they collectively define the organization of cells in the resulting embryos. The larval cell type-level of analysis can be treated as a compromise, allowing both gene expression analysis and avoiding the analysis of complex organ systems that were not yet set up in early larvae. Some cell types are directly related to the emergence of new organs during evolution. Shell secreting cells in molluscs are for instance at the origin of a morphological innovation that resulted in the highly successful evolutionary radiation of molluscs. One-to-many comparisons of gene expression data from individual cells could yield further insight into the evolutionary history of both the genetic

networks and the evolutionary identity of the vast variety of animals present today [81]. Although, to date very few examples exist indicating the conservation of cell morphology and gene expression over large evolutionary time periods, their existence should not be easily dismissed. Indeed, cells expressing distinct classes of light sensitive G protein-coupled receptors called opsins with correspondingly distinct downstream signaling cascades and subcellular organization have been observed in vertebrates and invertebrates. So-called "ciliary photoreceptor cells" possess "ciliary opsins" which trigger cyclic nucleotide gated ion channels, and upon activation result in hyperpolarization of the cell. These cells tend to concentrate their photosensitive proteins as the name indicates in primary cilia. On the other hand, "rhabdomeric photoreceptor cells" carry "rhabdomeric opsins" that trigger a signaling cascade which results in a depolarization of the cell and localize their opsins to microvillar structures called "rhabdomes". Both kinds of photoreceptive cells have been identified all over the animal kingdom, indicating linkage between the different characters and selective pressure to maintain both types of cells, despite a common function. The interchangeable aspect of these cells is furthermore underscored by the fact that a visual photoreceptor function is primarily carried out by ciliary-type photoreceptors in vertebrates and rhabdomeric-type photoreceptor cells in arthropods, molluscs and many invertebrates. Conversely a non-visual function (hypothetically circadian) function is carried out by rhabdomeric-type photoreceptor cells in vertebrates and hypothetically by ciliary-type photoreceptor cells in invertebrates[82, 83, 84].

Apprehending the Regulatory Cell

The question of the origin and evolution of complex organ systems has been puzzling scientists since Charles Darwin. As we have extensively discussed above, the problem of convergence and parallel evolution has hampered a proper examination of extensive organ homologies, even taking molecular evidence into account. For instance, despite the conservation of the molecular toolkit involved in animal development and organogenesis, the tissue- and organ-specific gene expression is not extensively conserved between the chordates *Ciona intestinalis* and zebrafish [85]. One can consider organs as mosaic of distinct cell types whose tangling prevents from an accurate recovery of a conserved regulatory profile.

To carry out such a comparison of gene expression profiles between lineages, it is pivotal to look back at the criteria evaluating the homology of cell types listed at the beginning of this chapter. Among them was the commonality of function and the concept of connectivity described by Lecointre. The principle of connectivity is very much amenable to gene expression studies as well as morphological considerations. Eric Davidson postulated that gene-regulatory networks and 'modules' underwent evolutionary pressure and selection as independent units rather than as individual genes [86]. Recently developments in single-cell transcriptomics then constitute invaluable directions to further assess the molecular conservation of gene expression at both the organismal and phylogenetic levels.

This of course raises the obvious question considering the vast diversity of animal developmental models of: at which stage should comparisons be carried out. The theory that young animal embryos often appear different, but eventually adopt a similar

aspect dubbed the ”phylotypic stage”, only to later diverge again in the adult forms, derives from a curious observation made by early 19th century German biologist Karl von Baer, who pointed out that he could not distinguish between two unlabeled larvae fixed in alcohol. This notion was later called the ”laws of von Baer” [87, 88], which claim that there is a ”formation of [...] general characters before species-specific characters” [87, 88]. The more recent ”hourglass” model”, similarly states that at early stages of development (cleavage, blastula) vertebrate embryos demonstrate a large diversity in terms of morphology, however at later stages, a so-called ”phylotypic” stage is observed, at which conserved morphological traits appear (Fig. 3). This stage has been hypothesized to take place approximately at the pharyngula stage. After this stage, the ”von Baer-like” divergence takes place again. Recently, molecular arguments in favor of the ”hourglass model” have been observed. Kalinka and colleagues measured differences in gene expression between various species of the fruitfly *Drosophila* overtime using DNA microarrays. Indeed the authors observed that the temporal gene-expression divergence among species is minimal around the ’extended germband’ stage, which is classically regarded as the phylotypic stage in insects [88, 89, 87]

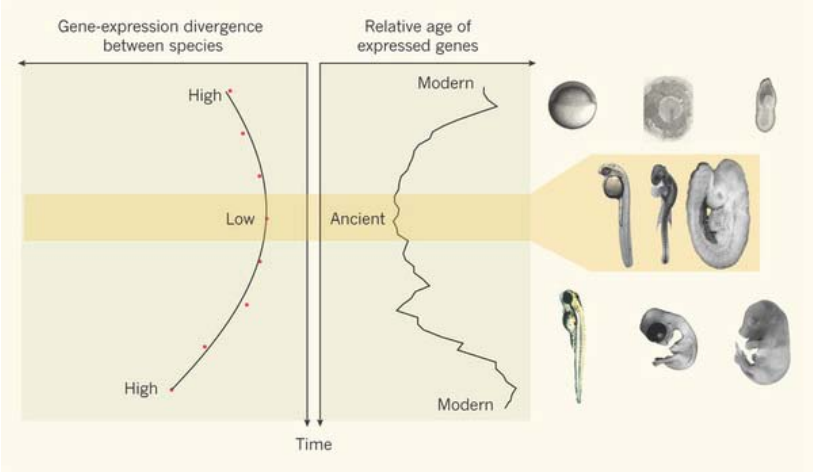


Figure 3: Mid-embryogenesis is marked by the phylotypic stage, a period of minimal anatomical divergence between species, as illustrated for vertebrate species by the orange band. This stage is now shown by Kalinka and colleagues to display minimal gene-expression divergence between *Drosophila* species (left curve), and to express the oldest gene set of the entire life cycle (right curve). The species depicted, left to right, are zebrafish, chick and mouse [88]

Conclusion

Since the dawn of mankind, natural philosophers and scientists have tried to categorize and understand the variability of life. The classification of the building blocks, the cells, has been a subject of much discussion. The outlined criteria pose unsurmountable difficulties if all these criteria are abided by, whilst recent progress in molecular biology techniques has opened doors to observe and characterize cells at an ever higher resolution.

In Chapter 1, I describe work performed in the lab of Dr. Carl Neumann under the supervision of Dr. Marcus Dekens, studying a very curious animal, *Danio rerio*, the zebrafish, whose every cell has taken up the function reserved to only a select few in most other organisms - that of light sensitivity. The light-dependent transcriptome of early larvae that do not yet possess a photoreceptive organ were analyzed. This work was published in 2010 in PLos One.

In chapters 2 to 4, I present work performed in the lab of Pr. Dr. Detlev Arendt, on the marine annelid worm *Platynereis dumerilii*. In order to gain insight into the "true and intelligible reason of all the Phenomena" as Hooke would have said, of this organism, I set out to characterize gene-expression of select larval cells. In order to obtain individual cells, methods to dissociate larval and adult *Platynereis* into individual cells most efficiently were established. This is presented in Chapter 2. Furthermore, as mentioned above, cell morphotypes in *Platynereis* have been described in great detail *in toto*, however how their morphology differs once the environmental factors and pressures affecting their cells are removed is not clear. In Chapter 3, an attempt at an morphological and immunohistochemical characterization of *Platynereis* cells is described. Once the morphological and immunohistochemical characterization of Platynereis larvae was assayed, this yielded the possibility to characterize the transcriptome of selected *Platynereis* cells. Furthermore, as *Platynereis* cells could be dissociated and collected, an attempt at cell culture and medium determination and transfection was made. This is described in Chapter 4.

Table 1: Number of cell types (reproduced from [44])

Phylum	Cell Morphotypes
Acanthocephala	12
Annelida	37
Arthropoda	42-90
Brachiopoda	34
Bryozoa	25
Chaetognatha	21
Chordata	38-215
Cnidaria	10
Ctenophora	17
Cycliophora	15
Echinodermata	41
Echiura	21
Entoprocta	13
Gastrotricha	23
Gnathostomulida	16
Hemichordata	16
Kinorhyncha	17
Loricifera	18
Mollusca	39
Myxozoa	3
Nematoda	14
Nematomorpha	8
Nemertea	35
Onychophora	30
Orthonecta	3
Phoronida	23
Placozoa	4
Platyhelminthes	20
Pogonophora	20
Porifera	4
Priapulida	20
Rhombozoa	4
Rotifera	15
Sipuncula	25
Tardigrada	18
Urochordata	38

Chapter 1

Thyrotroph Embryonic Factor Regulates Light-Induced Transcription of Repair Genes in Zebrafish Embryonic Cells

1.1 Introduction

The circadian clock times a wide variety of biological processes, ranging from behavior and metabolism to DNA replication. The core clock mechanism consists of a self-sustaining transcription-translation auto-regulatory feedback loop. The heterodimer composed of CLOCK (CLK) and Brain Muscle ARNT-Like (BMAL) binds to enhancers upstream of the Period (Per) and Cryptochrome (Cry) genes to initiate their transcription. The repressers PER and CRY interact with the CLK:BMAL heterodimer and thereby down-regulate their own expression. In mammals a centralized clock, which resides in the hypothalamic suprachiasmatic nucleus (SCN) and is innervated by the retina, controls temporal adaptation. This master clock relays a signal to the peripheral clocks thereby setting their phase. Zebrafish rely on peripheral circadian clocks that are directly entrained by light, indicating a high degree of cell autonomy [90, 91]. How the light signal is detected in these cells, and how seemingly independent processes are integrated with these peripheral circadian clocks remains unresolved. The regulation of several light dependent processes is mediated by basic leucine zipper (bZip) transcription factors in mammals. These transcription factors transactivate target genes by binding as homo or heterodimers to the response elements in their promoters [92]. The FOS-JUN subfamily of bZip transcription factors function in light activation of neurons in the SCN [93]. The members of the proline- and acidic amino acid-rich (PAR) subfamily, TEF (Thyrotroph Embryonic Factor), DBP (D-site Binding Protein) and HLF (Hepatocyte Leukaemia Factor), play a role in the regulation of circadian output processes [94]. Furthermore, the cAMP Response Elements (CRE),

which are regulated by the CRE Binding (CREB) subfamily, are also under circadian clock control [95]. Since zebrafish cells can directly detect light, this organism offers the possibility to study how light affects peripheral cells. To identify the genes that exhibit light dependent transcription in zebrafish embryos we used microarrays to screen over 14,900 transcripts, aiming to gain an insight into the regulatory networks driving these genes. Here we demonstrate that the PAR bZip transcription factor Tef α plays a key role in the regulation of light-induced genes that function in DNA repair and in counteracting the adverse effects of reactive oxygen species.

OPEN ACCESS Freely available online

PLoS one

Thyrotroph Embryonic Factor Regulates Light-Induced Transcription of Repair Genes in Zebrafish Embryonic Cells

Daria Gavriouchkina, Sabine Fischer, Tomi Ivacevic, Jens Stolte, Vladimir Benes*, Marcus P. S. Dekens*

Genomics Core Unit, European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany

Abstract

Numerous responses are triggered by light in the cell. How the light signal is detected and transduced into a cellular response is still an enigma. Each zebrafish cell has the capacity to directly detect light, making this organism particularly suitable for the study of light dependent transcription. To gain insight into the light signalling mechanism we identified genes that are activated by light exposure at an early embryonic stage, when specialised light sensing organs have not yet formed. We screened over 14,900 genes using micro-array GeneChips, and identified 19 light-induced genes that function primarily in light signalling, stress response, and DNA repair. Here we reveal that PAR Response Elements are present in all promoters of the light-induced genes, and demonstrate a pivotal role for the PAR bZip transcription factor Thyrotroph embryonic factor (Tef) in regulating the majority of light-induced genes. We show that *tefβ* transcription is directly regulated by light while transcription of *tefα* is under circadian clock control at later stages of development. These data leads us to propose their involvement in light-induced UV tolerance in the zebrafish embryo.

Citation: Gavriouchkina D, Fischer S, Ivacevic T, Stolte J, Benes V, et al. (2010) Thyrotroph Embryonic Factor Regulates Light-Induced Transcription of Repair Genes in Zebrafish Embryonic Cells. PLoS ONE 5(9): e12542. doi:10.1371/journal.pone.0012542

Editor: Bruce B. Riley, Texas A&M University, United States of America

Received July 5, 2010; **Accepted** July 27, 2010; **Published** September 7, 2010

Funding: This work was funded by the European Molecular Biology Laboratory. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: marcus.dekens@gmail.com (MPSD); benes@embl.de (VB)

Introduction

The daily sunlight-darkness cycle is one of the most extreme and repetitive variations in environmental conditions that organisms are exposed to. The necessity to adapt gave rise to light detection mechanisms and a circadian clock, which times a variety of physiological and cellular processes. Several circadian components and DNA damage response proteins are closely related. For example the Cryptochrome (Cry) proteins that transduce the light signal to the circadian clock, either as photoreceptors or as transcriptional repressors, belong to the same family of flavin-containing proteins as the DNA repair enzyme Photolyase (Phr) [1]. Photolyase may have been the first existing light-detecting molecule [2]. Pittendrigh [3], and thereafter Gehring and Rosbash [4], proposed that a circadian oscillator was established and coupled to these blue light photoreceptors to anticipate damage. Interestingly, a link between the clock and nucleotide excision repair was recently reported [5]. Thus light detection and the circadian clock may have originated to avoid DNA lesions [6]. How the light signal is detected in cells, and how seemingly independent processes are integrated with the circadian clock remains unresolved.

In mammals a centralised clock, which resides in the hypothalamic suprachiasmatic nucleus (SCN) and is innervated by the retina, controls temporal adaptation. This master clock relays a signal to the peripheral clocks thereby setting their phase. Zebrafish rely on peripheral circadian clocks that are directly entrained by light, indicating a high degree of cell autonomy [7,8]. The core clock mechanism consists of a self-sustained transcription-translation auto-regulatory feedback loop [9]. The heterodimer composed of Clock (Clk) and Brain muscle ARNT-like (Bmal) binds to enhancers upstream of the *period* (*per*) and *cryptochrome* (*cry*) genes to initiate their transcription. The repressors Per and Cry interact with the Clk:Bmal heterodimer and thereby down-regulate their own expression.

In zebrafish light-induced activation of the mitogen-activated protein kinase (MAPK) pathway has been shown to regulate *per2*, *cry1a* and *64phr* expression [10,11]. Thus the same light-signalling pathway controls light dependent UV tolerance and circadian clock entrainment. Also several basic leucine-zipper (bZip) transcription factors play a role in mediating the regulation of light dependent processes. The AP-1 (Activator Protein-1) complex, a heterodimeric protein composed of the bZip transcription factors c-Fos and c-Jun, exhibits light dependent transcription. The transcription factor AP-1 is regulated by the MAPK signal transduction pathway, and is an important component of the mammalian UV response [12–14]. Furthermore, TEF (Thyrotroph Embryonic Factor), DBP (D-site Binding Protein) and HLF (Hepatocyte Leukaemia Factor), belonging to the proline- and acidic amino acid-rich (PAR) bZip subfamily, mediate the regulation of metabolic detoxification and are under circadian clock control in mouse [15]. These transcription factors transactivate target genes by binding as homo or heterodimers to the PAR Response Elements (PARRE) in their promoters [16]. However, signal-induced gene expression is not mediated by linear signal transduction pathways targeting a single response element,

but involves networks of signalling molecules and transcription factors targeting multiple control elements that cooperate to regulate gene transcription.

This study aims to attain insight into the light signalling mechanism by screening for genes that are light activated and subsequently identifying common regulatory networks driving these genes. The zebrafish is a particularly suitable organism for studying light dependent transcription due to the ability of each cell to directly detect light. We analysed over 14,900 transcripts using the Affymetrix micro-array GeneChip and identified 19 genes that exhibit light-induced transcription. Here we demonstrate by computational promoter analysis in combination with knock down experiments that the PAR bZip transcription factor Tef plays a key role in the regulation of light-induced genes that function primarily in DNA repair and in counteracting the adverse effects of reactive oxygen species.

Results and Discussion

A screen for genes that display light dependent transcription

Zebrafish embryos become light responsive around 5 hours post fertilisation (h.p.f.), and light sensitivity increases during the following 4 hours [17,18]. The far from fully differentiated cells at this early stage of development are an appealing model as one can select for genes that display light dependent transcription before specialised light sensing organs have formed. We screened with the Affymetrix micro-array GeneChip for light regulated genes by comparing the differential expression between embryos exposed for the first 9 hours of development to light (LL) and siblings maintained in constant darkness (DD) (Figure 1A, Table 1). All genes that have a differential expression of 2-fold or more on the micro-array chip were validated by quantitative Polymerase

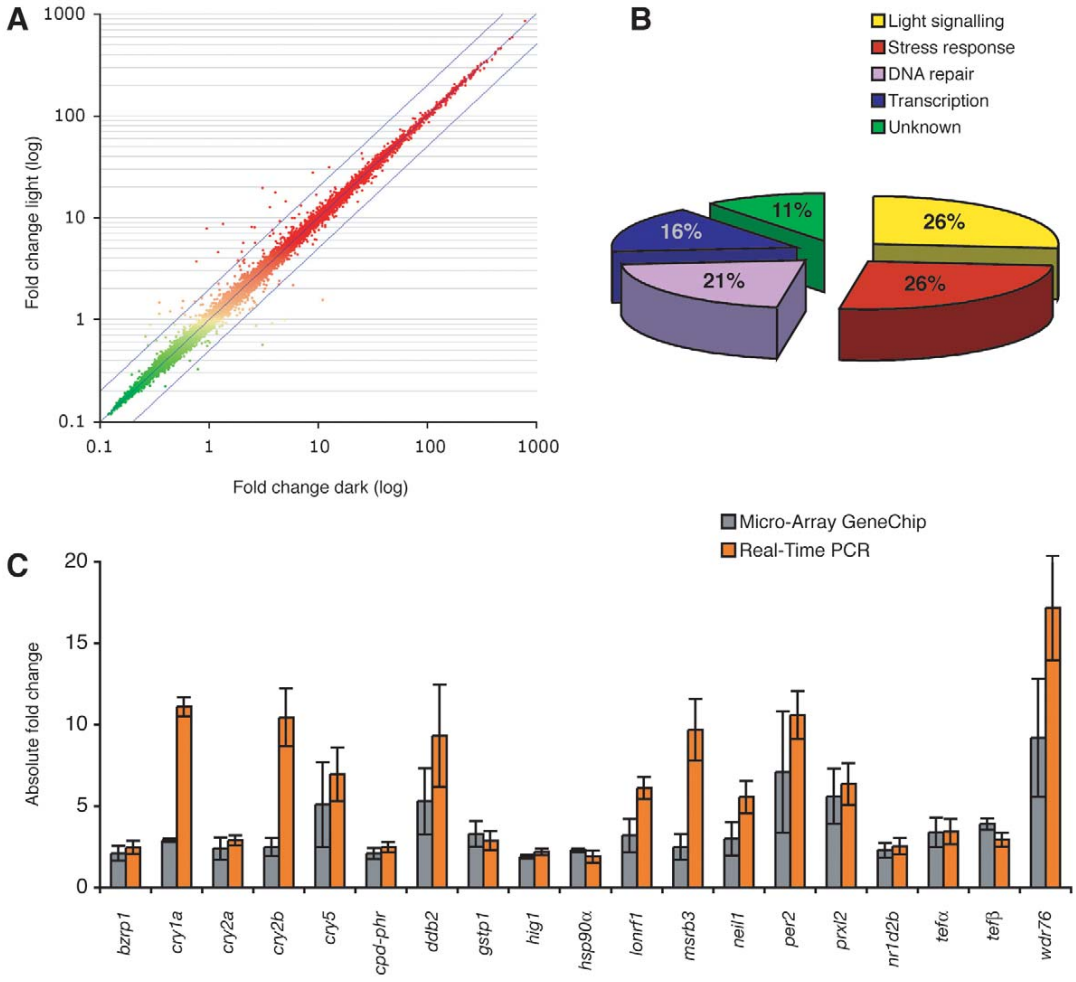


Figure 1. Screen for genes that display light-induced transcription. (A) Scatter plot showing the fold change in transcript level between embryos (9 h.p.f.) that were exposed to light and siblings maintained in darkness. Each dot represents one transcript of 14,900 genes screened. The reliability of the data is indicated by a green to red colour scale, with only the red dots representing transcripts that have a trustworthy differential expression. The outer blue lines demarcate the 2.0 fold boundaries when related to the average (central blue line). (B) Pie chart representing the ratios of the different processes in which light-induced genes function. (C) Validation by qPCR (n = 7) of the 19 light-induced transcripts that were identified by the Affymetrix micro-array GeneChip (n = 3, fold change >2). Grey bars indicate micro-array fold change and orange bars indicate qPCR fold change. Error bars indicate the standard deviation in all experiments. The qPCR fold changes shown were normalised using DD transcript levels, and differences between samples were corrected with β-actin mRNA levels.
doi:10.1371/journal.pone.0012542.g001

Table 1. Genes that display light-induced transcription.

Gene Symbol	Gene Name	GeneChip Fold Change	Real-Time PCR Fold Change	Process	GenBank No.
wdr76	WD40-repeat protein 76	9.2±3.6	17.2±3.2	DNA repair	XM_693494
cry1a	cryptochrome 1a*	2.9±0.1	11.1±0.6	Light signalling	NM_131789
per2	period 2 [35,36]	7.1±3.7	10.6±1.5	Light signalling	NM_182857
cry2b	cryptochrome 2b	2.5±0.6	10.5±1.8	Light signalling	NM_131792
msrb3	methionine sulfoxide reductase B3	2.5±0.8	9.7±1.9	Stress response	NM_001002094
ddb2	UV damage DNA binding protein 2	5.3±2.0	9.3±3.1	DNA repair	NM_001083061
cry5	cryptochrome 5 [17]	5.1±2.6	7.0±1.6	Light signalling	NM_131788
prxl2	peroxiredoxin-like 2	5.6±1.7	6.4±1.3	Unknown	NM_213313
lonrf1	LON-protease ring finger 1	3.2±1.0	6.1±0.7	Unknown	XM_684170
neil1	nei endonuclease VIII-like 1	3.0±1.0	5.6±1.0	DNA repair	NM_200283
tefα	thyrotroph embryonic factor α	3.4±0.9	3.4±0.8	Transcription	NM_131400
gstp1	glutathione S-transferase p1	3.3±0.8	2.9±0.6	Stress response	NM_131734
tefβ	thyrotroph embryonic factor β	3.9±0.3	2.9±0.4	Transcription	U96848
cry2a	cryptochrome 2a	2.4±0.7	2.9±0.3	Light signalling	NM_131791
bzrp1	benzodiazepine receptor 1	2.1±0.5	2.5±0.4	Stress response	NM_001006032
cpd-phr	cpd-photolyase-like	2.1±0.3	2.5±0.3	DNA repair	NM_201064
nr1d2b	nuclear receptor 1D2b	2.3±0.5	2.5±0.5	Transcription	NM_131065
hig1	hypoxia induced gene 1	1.9±0.1	2.2±0.2	Stress response	NM_200100
hsp90α	heat shock protein 90α	2.3±0.1	1.9±0.4	Stress response	NM_001045073

*Note that the light-induced gene products could function in several different processes, for instance Cry1a also plays a role in stress response [24].

doi:10.1371/journal.pone.0012542.t001

Chain Reaction (qPCR) (Table 1, Figure 1C), thereby confirming 19 transcripts to be significantly induced by light in zebrafish early embryonic cells. Interestingly, this light-induced gene set shows no similarity with light-induced transcripts in the mouse SCN [14], the specialized direct light responsive cells in a mammal. Furthermore, the genes that are suppressed by light, apart from the circadian clock regulated gene *egln3* [19], could not be reproduced by qPCR (Table S1). The light-induced genes identified function in DNA repair, stress response, and light signalling (Figure 1B, Table 1).

A large proportion of the light-induced genes belong to the family of Cryptochromes. Many responses to light are mediated by CRYs [20,21], a subset acts as photopigments [10] while others play a role in light signalling or may take part in the core circadian oscillator, as is the case in mammals. Seven *cry* genes have so far been reported in zebrafish [22]. In particular, zebrafish Cry1a has been shown to reset the clock [23], and also has been reported to play a role in oxidative stress response [24].

Several genes with a function in DNA repair are expressed at high levels in light exposed embryos, including the nucleotide excision repair gene *ddb2* and its homologue *wdr76*. In humans the WDR76 protein is associated with the CUL4-DDB1 ubiquitin ligase complex [25]. Interestingly, an increase in *WDR76* expression is observed during DNA replication [26], which is under circadian clock control in zebrafish [27,12]. We also observed an increase in transcript level of the DNA glycosylase Neil1, which initiates the first step in base excision repair by cleaving bases damaged by oxygen radicals [28]. Furthermore we demonstrate light-induction of a photolyase-like gene, *cpd-phr*, that removes cyclobutane pyrimidine dimers [29]. *ap-1* is light induced in adult zebrafish [12], however it was not detected by the micro-array chip at an early stage of development.

Reactive oxygen species have been reported to induce the transcription of direct light responsive genes in zebrafish [24]. We show here that the expression levels of many stress response genes are elevated during light exposure, such as *msrb3*, which has a function in the repair of oxidized proteins [30], and *gstp1*, which has a catalytic function in the detoxification of electrophiles thereby neutralizing products of reactive oxygen species [31]. The light-induced peripheral *bzrp1* gene opposes apoptosis during oxidative stress by controlling mitochondrial membrane permeability [32]. Also the stress response gene *hig1* has an anti-apoptotic function [33]. Furthermore we observe higher transcript levels of *hsp90α*, its gene product being essential for refolding of denatured proteins [34].

We applied *in situ* hybridization to several of the light-induced genes during retinal development to determine if any of the genes display enriched expression in specialized light sensing tissues (Figure 2A–J). Published *in situ* patterns of light-induced transcripts were not examined [17,35,36]. All transcripts are ubiquitously expressed, and the transcripts of *wdr76*, *cry1a*, *msrb3*, *neil1*, and *tef* show higher levels in the retina, and the *hsp90α* and *tef* transcripts are present at a higher level in the pineal (Figure 2A, 2B, 2C, 2F, 2G, 2J).

The micro RNAs (miRNAs), a class of small non-coding transcripts, play a key role in post-transcriptional gene regulation. Since the zebrafish genome array GeneChip did not include the detection of miRNAs, we extended the screen by testing reported oscillating mouse and *Drosophila* miRNAs for rhythmic expression in zebrafish. Animals were entrained on light-dark (LD) cycles and samples were taken during the first four days post fertilisation. We show by qPCR that miR132 and miR219 are rhythmically expressed during zebrafish development ($p<0.05$; Figure 3A and 3B). Interestingly, the miRNAs show peak and trough transcript levels at opposite zeitgeber times (zt) as reported for mouse [37].

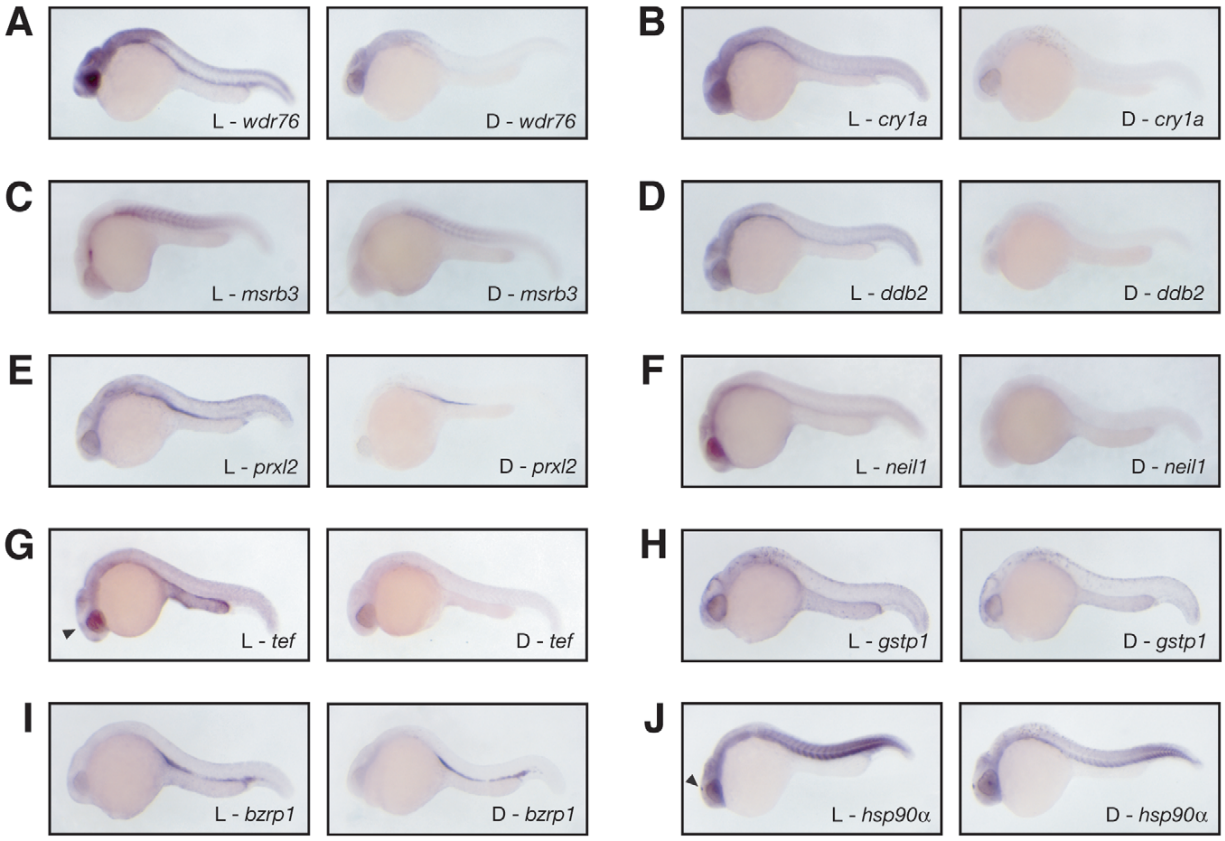


Figure 2. Light-induced transcripts are expressed ubiquitously. (A) In situ hybridizations of embryos (at 25 h.p.f.) that were exposed to light (left) or maintained in darkness (right) for the probe against wdr76, (B) cry1a, (C) msrb3, (D) ddb2, (E) prxl2, (F) neil1, (G) tef, (H) gstp1, (I) bzrp1, and (J) hsp90α. All light-induced transcripts are expressed ubiquitously at the early stages of zebrafish development. For most transcripts a gradient is observed with the highest level of expression at the anterior. The wdr76, cry1a, msrb3, tef, and neil1 transcripts are present at substantially higher levels in the retina. The hsp90α and tef transcripts show a distinctive presence in the pineal (indicated by arrowhead).
doi:10.1371/journal.pone.0012542.g002

Tef mediates the regulation of light-induced transcription

Given that Tef and Nr1D2b are the only transcription factors identified by the screen, we hypothesized whether they could play a central role in the activation of light-induced transcription. The *tef* gene encodes a PAR bZip transcription factor that binds to PARREs (PAR Response Elements) in the promoters of target genes, and is under circadian clock control in mouse [38]. The *nr1d2b* (*rev-erbβ*) gene encodes an orphan nuclear receptor that binds to ROR (RAR-related Orphan Receptor) elements in the promoters of target genes, and is a paralog of a regulatory component of the circadian clock (*nr1d1* or *rev-erbα*) [39]. To further investigate their role, we used phylogenetic promoter analysis software (GenomatixSuite) to screen for regulatory sequences in the light-induced gene set. This predicted the presence of PAR response elements (puTTApyGTAApy) in the promoters of all light-induced genes (Figure 4), however only a few promoters include E-box and ROR elements. Since all promoters contain PARREs, this points to Tef being an evident candidate for the regulation of light-induced transcription.

To verify the computational data, we next tested whether knock down of *tef*, by injecting morpholino-modified anti-sense oligonucleotides in light exposed embryos, reduces the transcript levels of light-induced genes. Two zebrafish isoforms have been reported, *tefα* and *tefβ*, which are transcribed from separate promoters [40]. Knock down of *tefα* results in strongly reduced transcript levels of: *bzrp1*, *cpd-phr*, *cry1a*, *cry2b*, *cry5*, *ddb2*, *gstp1*, *lonrf1*, *msrb3*, *neil1*, *per2*, *prxl2*, and *wdr76* ($p<0.05$; Figure 5A). Interestingly, a zebrafish *per2* promoter study showed this gene to be regulated by Tef [41], and thus supports the data presented here. Furthermore, a reduced *gstp1* transcript level has been reported in *Hlf/Dbp/Tef* triple knock out mice [15]. *tefα* knock down does not significantly affect miR132 and miR219 expression levels, consistent with the absence of PARREs in their promoters (data not shown). Knock down of *tefβ* mildly reduced expression of: *cry1a*, *ddb2*, *hig1*, *per2*, and strongly reduced expression of: *cry2b*, *lonrf1*, *msrb3*, and *prxl2* ($p<0.05$; Figure 5B). Double knock down of *tefα* and *tefβ* produces the same effect as single *tefα* knock down, but results in even lower levels of *cry2b* and *ddb2* ($p<0.05$; Figure 5C). The reduced transcript level in *tef* knock down embryos is consistent with direct regulation by transcriptional activation, as suggested by the PARREs present in the promoters of the light-induced genes. However, it cannot be ruled out that Tef indirectly regulates these genes. Since the transcript levels of light regulated genes in *tef* knock down LL embryos are rarely reduced to their DD levels, other factors must also play a role in controlling light dependent processes. Importantly, several other members of the bZip family have the capacity to bind the PARRE. In all knock down experiments, *tef* mRNA levels are not affected, implying that *tef*

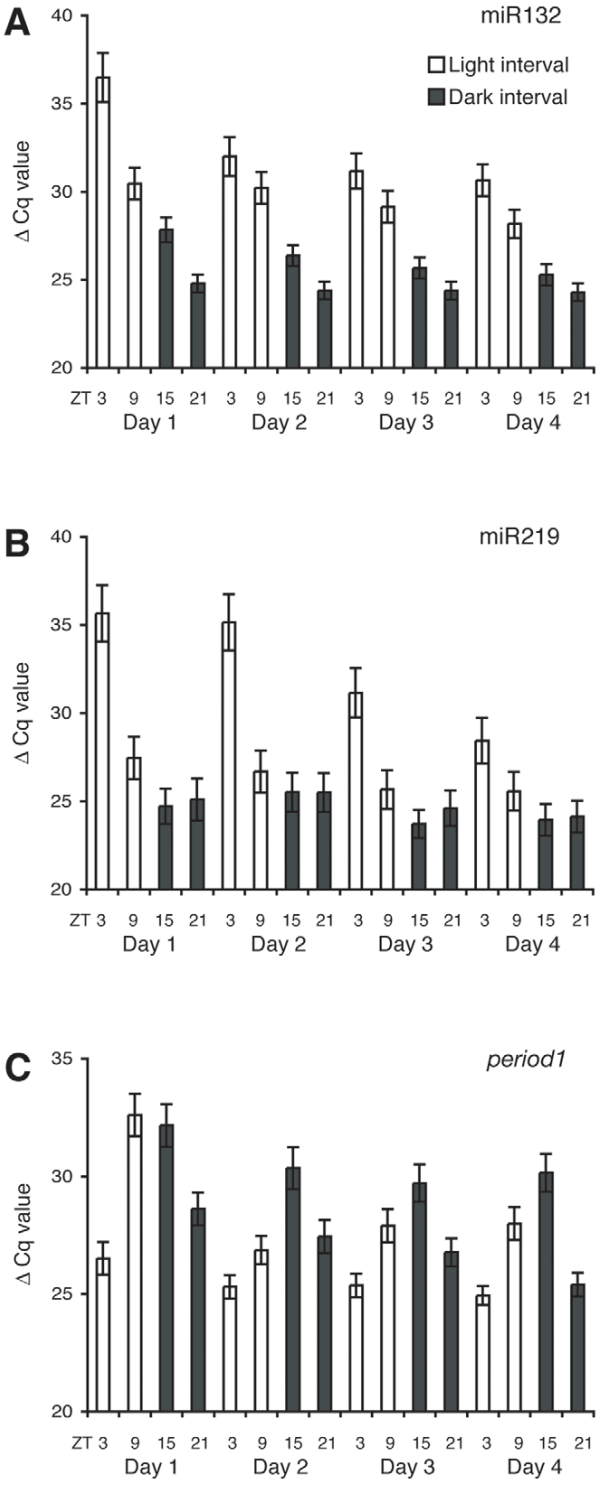


Figure 3. miR219 and miR132 temporal expression pattern. (A) qPCR analysis showing the temporal oscillation of miR132 and (B) miR219 transcription during the first four days of development in embryos raised under a 12:12 LD cycle. (C) Expression of *period1* under the same conditions. White bars indicate the light and black bars the dark intervals. Note that low cycle quantification (Cq) values indicate high transcript levels and vice versa.
doi:10.1371/journal.pone.0012542.g003

does not regulate itself although PARREs are present in the *tef* promoters. To confirm the knock down data we over expressed *tefα* by microinjecting mRNA in DD embryos, this results in a significant increase in the levels of: *bzrp1*, *cpd-phr*, *cry2a*, *gstp1*, *hig1*, *lonrf1*, *msrb3*, *neil1*, and *prxl2* ($p<0.05$; Figure 5D). Thus we demonstrate *tefα* to play a crucial role in the regulation of many light-induced genes, while *tefβ* has a less prominent function.

Tefα is under circadian clock control

Based on the regulation of *tef* in mouse, we hypothesised whether the two isoforms of Tef are under circadian clock control. We entrained zebrafish larvae to LD cycles for the first four days of development and analysed *tef* mRNA levels. This revealed that *tefα* and *tefβ* transcription oscillate during development, showing peak and trough transcript levels at opposite zt's as reported for mouse [42]. The *tef* transcript level reaches its peak during the light period, as expected from its role in mediating the regulation of light-induced transcription (Figure 6A and 6B, note that low cycle quantification values indicate high transcript levels and vice versa). To investigate if *tef* is under circadian clock control, larvae were subjected to LD cycles during the first 3 days of development followed by DD over the consecutive days. A rhythm of *tefα* transcription is observed on the days following the LD cycles (Figure 6C). The observed light entrainment reflects *tefα* regulation through an oscillator. As it is currently technically not feasible to determine how *tefα* is regulated at the earliest stages of development, the possibility exists that *tefα* is initially directly induced by light. The difference in *tefβ* transcript levels is not significant on the first and second day in DD ($p>0.05$; Figure 6D), thus suggesting a direct light driven mechanism during the first days of development, however this gene may later on become under circadian clock control. The role for Tefα in the regulation of stress response and DNA repair genes may imply the circadian clock in their regulation. Gachon and colleagues [15] demonstrated a role for the circadian transcription factors TEF, DBP, and HLF in the regulation of various processes in mouse, including metabolic detoxification. TEF, DBP, and HLF are expected to regulate different target genes as they have different target promoter preferences [38,42]. Triple knock out of all PAR bZip family members in mouse results in epilepsy and accelerated ageing, but does not lead to developmental defects [43]. In the mouse embryo *Tef* expression is only present in the anterior pituitary [44], while in the zebrafish embryo it is ubiquitously transcribed. This may imply centralized regulation during development for the mouse *Tef* gene in contrast to the zebrafish embryo.

Light-induced UV tolerance

Zebrafish spawn at light onset in shallow rivers, thus the embryos are already exposed to sunlight at the earliest stage of development. Here we demonstrate that visual light induces genes that function in light signalling, stress response or DNA repair in zebrafish embryonic cells. We used a light source that only emits low radiation in the UV-A and no radiation in the harmful UV-B and UV-C range of the spectrum (Figure S1), and the embryos were separated from the source by a 4 mm Perspex plate and at least 5 cm water. Therefore it is not possible that the identified DNA repair genes were induced by UV damage. Interestingly, embryos maintained in constant darkness and subsequently UV-irradiated show a lower survival rate when compared to UV-treated embryos that were previously exposed to light [17]. The superior survival in the latter case can be explained by the increase in transcription of stress response and DNA repair genes. Several mechanisms that relay the light signal directly or via the circadian

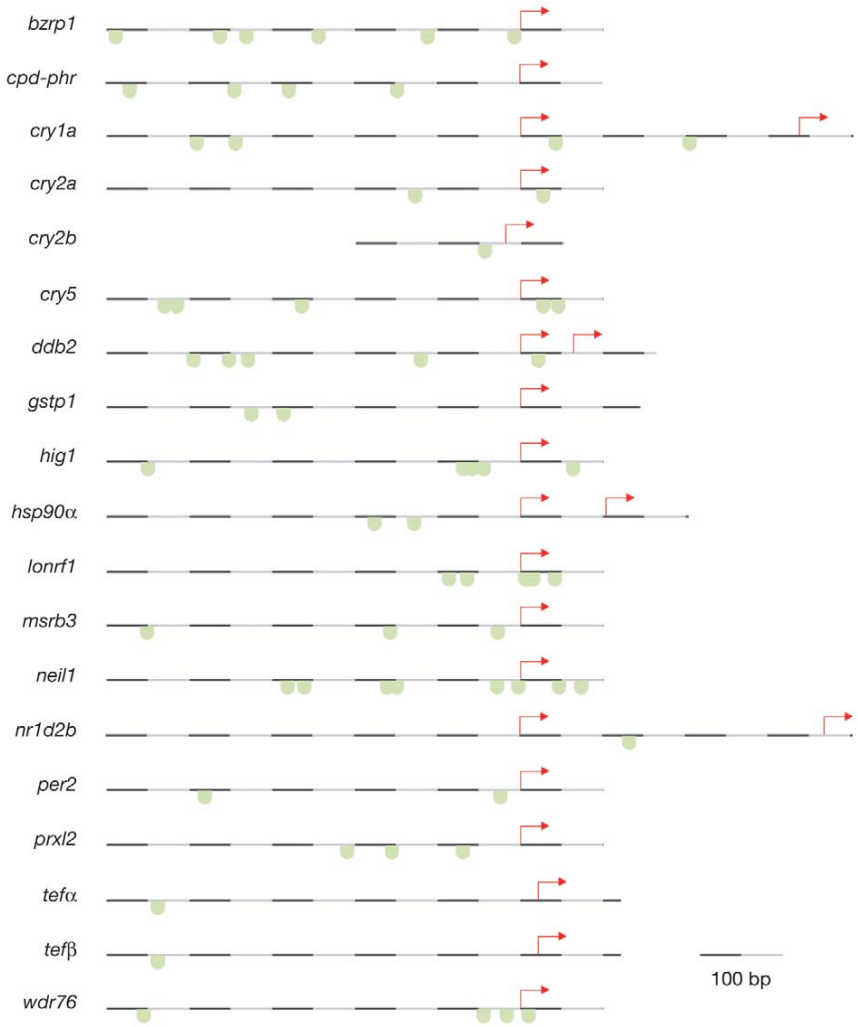


Figure 4. All promoters of the light-induced genes contain PAR elements. Genomatix software was used to predict PAR response elements in a 500 bp region upstream of the start codon in the promoters of all light-induced genes. Since the untranslated region of *cry2b* is not present in the Genomatix database this gene was analysed separately. Start codons are indicated with red arrows, and the locations of predicted PAR elements are marked with green boxes.
doi:10.1371/journal.pone.0012542.g004

clock can be envisaged. Light-induced activation of the MAPK signalling pathway plays an important role in 64Phr expression [11], which has the capacity to repair DNA in zebrafish. Cry1a can convey the light signal to the circadian clock as it binds directly to the core clock components Clk and Bmal [23,45]. We demonstrate *tefβ* expression to be directly driven by light in the zebrafish embryo, and *tefα* transcription to be under circadian clock control at later stages of development. Although the *tef* genes seem to be differently regulated, it is most likely that both *tef* genes are initially directly regulated by light and later on become under the control of the circadian clock. In addition, it is plausible that *tef* is regulated through the circadian clock as well as a direct light pathway. Since more DNA lesions are induced when cells are exposed to sunlight than during the night, the number of mutations could most likely be reduced if DNA damage were anticipated. Indeed DNA excision repair was demonstrated to be under circadian clock control in mammals [5]. We show here that the Tef transcription factors play a pivotal role in regulating DNA damage and stress response processes in the zebrafish embryo, and we suggest their involvement in light-induced UV tolerance. At later stages Tef could play a key role in coupling the circadian clock to repair processes, as several of the light-induced repair genes are rhythmically transcribed during development. Considering its crucial function in regulating various processes, ranging from metabolic detoxification to DNA repair, this transcription factor will be of high interest for future research.

Materials and Methods

Experimental setup

Zebrafish were raised following standard protocols [46]. Embryos were transferred to tissue culture flasks and submerged in thermostatically controlled water baths to maintain a constant temperature of 28°C. The setup is positioned within a light-sealed and air conditioned box. Embryos were illuminated with a compact fluorescent lamp (140 $\mu W/cm^2$, Figure S1) connected to a timer. The spectrum of the light source was determined using a fiber optic spectrometer (USB2000, OceanOptics Inc).

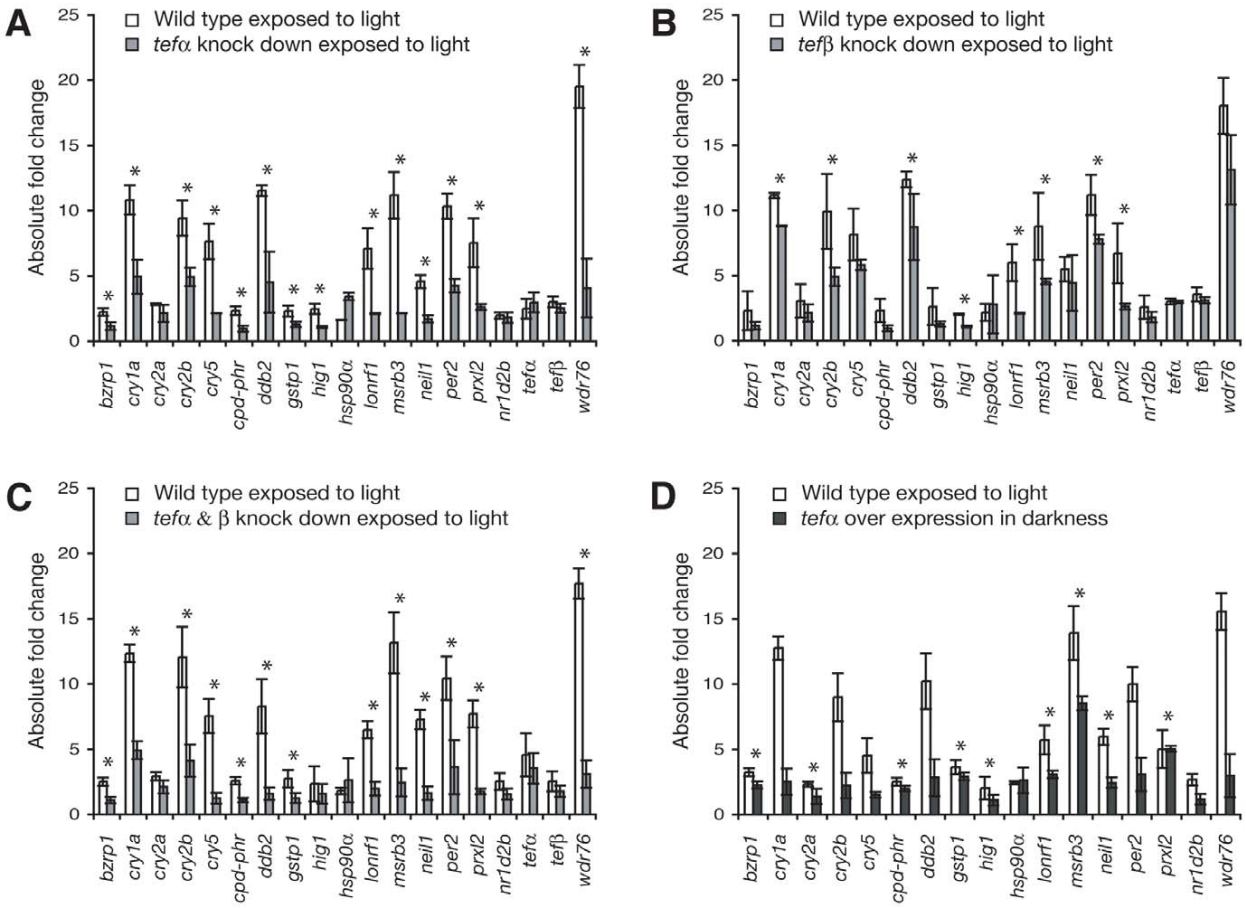


Figure 5. Tef regulates light-induced transcription. (A) Transcript level analysis by qPCR after 9 hours of light exposure in morpholino microinjected *tefα* knock down embryos (grey bars) compared to untreated embryos (white bars). Asterisks indicate significant difference in expression level. (B) Experiment as in A for morpholino-mediated knock down of *tefβ* in light exposed embryos. Demonstrating the reduced effect of *tefβ* knock down on the levels of most light-induced transcripts when compared with *tefα* knock down. (C) Morpholino-mediated double knock down of *tefα* and *tefβ* in light exposed embryos matches the *tefα* knock down result. (D) Embryos microinjected with *tefα* mRNA and directly transferred to DD. *tef* over expression results in elevated transcript levels when compared to untreated embryos maintained in DD. Fold changes were normalized with DD transcript levels, thus the knock down, over expression, and wild type light exposed transcript levels are compared to the wild type expression level in DD, which is set at zero on the Y-axis within each bar. Differences between samples were corrected with *β-actin* mRNA levels. These data clearly demonstrate that Tef mediates the regulation of light-induced transcription.
doi:10.1371/journal.pone.0012542.g005

Micro-array hybridization assay

Differential gene expression was determined by comparing two conditions: one group of sibling embryos was exposed to light, while the other group of siblings was transferred to constant darkness within 30 min after being laid. Embryos were harvested at 9 h.p.f. (ZT/CT9), and total RNA was extracted from 50 embryos per group using TRIzol Reagent (Gibco BRL) according to the manufacturer's instructions. From each condition 3 μg total RNA was used to synthesize biotinylated cRNA according to the one-cycle protocol, followed by hybridization to the Affymetrix GeneChip Zebrafish Genome Array. All procedures were conducted using Affymetrix equipment, protocols, and GeneChip Operating Software. GeneSpring GX7.3 software (Agilent Technologies) was used for CEL file data analysis. The MAS5 algorithm was applied for condensation, and median normalization was used. The micro-array result presented is the average of three independent experiments. All data is MIAME compliant and deposited in the MIAME database. Transcripts are considered differentially expressed when the average change was 2 fold or more. GenomatixSuite (Genomatix) software was used for subsequent phylogenetic promoter analysis. The light-induced and light-suppressed gene sets were separately analysed. The analysis was performed with selected promoter elements and limited to the first 500 bps upstream of the start codon.

Quantitative PCR analysis

cDNA was obtained by transcribing 1 μg of total RNA using QuantiTect reverse transcription components (Qiagen). Absolute levels of transcript were determined with fluorescence based Real-Time PCR using Sybr Green PCR Master Mix and thermocyclers from Applied Biosystems (AB). Primers were designed to generate amplicons that cross exon junctions to eliminate contamination through genomic DNA amplification (Universal Probe Library software from Roche; http://www.roche-applied-science.com/sis/rtpcr/upl/ezhome.html). A list of primer sequences for all transcripts is given in Table S2. qPCR was performed using the following thermal cycling parameters: 95°C for 10 min, followed by 40 two-step cycles of 95°C for 15 sec and 60°C for 1 min. All

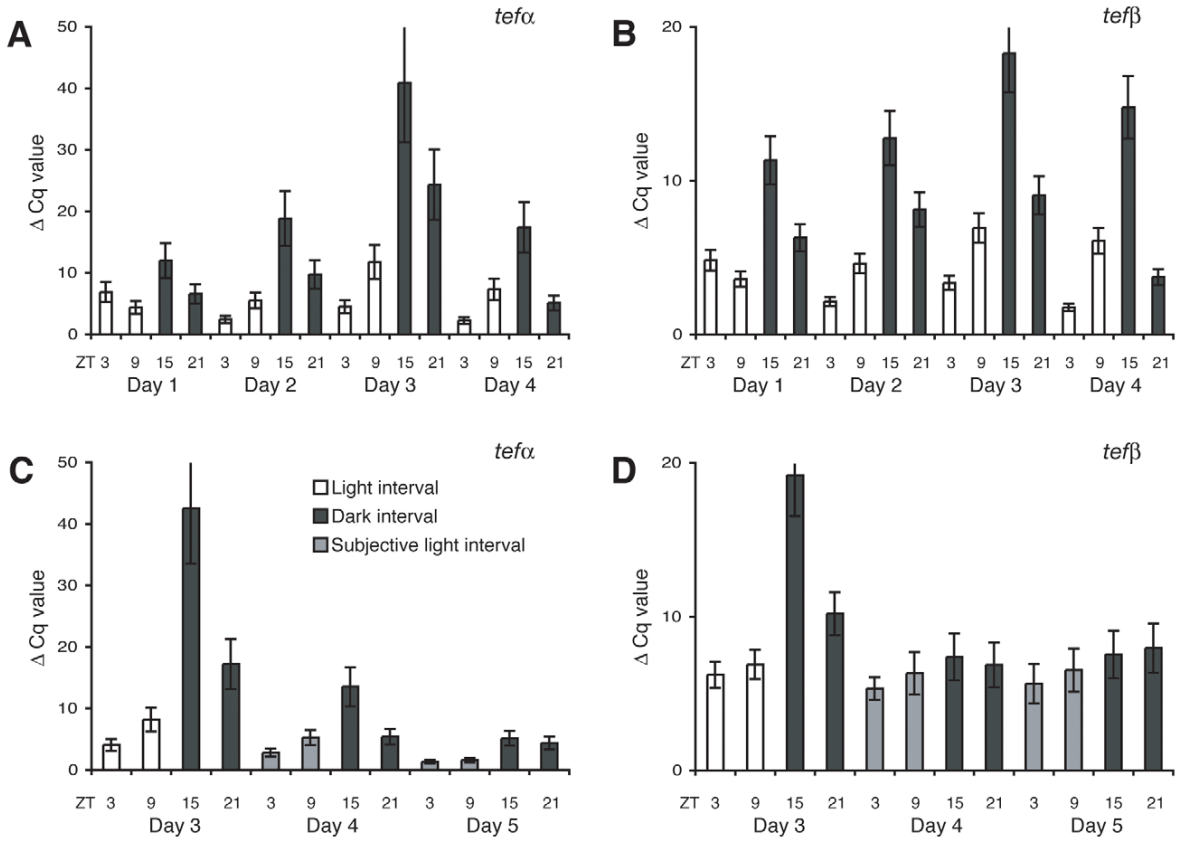


Figure 6. Transcription of *tefα* is under circadian clock control. (A) qPCR analysis showing the temporal transcript levels of *tefα* and (B) *tefβ* during the first four days of development in embryos raised under a 12:12 LD cycle. White bars indicate the light and black bars the dark intervals. (C) *tefα* mRNA levels on days 3, 4 and 5 in embryos entrained to LD cycles for the first 3 days followed by DD. Grey bars indicate the subjective light interval. The continuation of rhythmic expression in DD demonstrates that *tefα* transcription is regulated through an oscillator. (D) *tefβ* mRNA levels under the same experimental conditions as C. In all experiments the differences between samples were corrected with *β-actin* mRNA levels. Note that low Cq values indicate high transcript levels and vice versa.
doi:10.1371/journal.pone.0012542.g006

qPCR reactions were carried out in duplicate so that average cycle quantification values could be obtained. The absolute fold change was determined by normalising the level of transcription with the corresponding level in DD, and both levels were corrected for random errors with the *β-actin* level. The abundance of miRNA was also determined by qPCR. miRNAs were transcribed from 10 ng of total RNA using the TaqMan MicroRNA Reverse Transcript Kit (AB) and the human primers and probes for miR-132 [5′-UAACAGUCUACAGCCAUGGUCG-3′] and miR-219 [5′-UGAUUGUCCAAACGCAAUUCU-3′] (AB), followed by Real-Time PCR using the TaqMan Universal PCR Master Mix (AB) as indicated by the manufacturer. The absolute fold change was calculated using the comparative Δ(ΔCq) method (Relative Expression Software Tool) [47], and for all other experiments ΔCq was applied.The significance of the difference observed between two treatments within one experiment was determined with the Bayesian t-test.

In situ hybridization

In situ hybridisations were performed with anti-sense RNA fragments according to standard protocols. Probe synthesis was conducted with the components of the DIG RNA Labelling Kit (Roche). Embryos were fixed and subjected to methanol (Merck) and proteinase K (Roche) steps to enhance probe absorption. Embryos were hybridised with probe at 67°C overnight, followed by washing and labelling with sheep α-DIG AP-coupled Fab fragments (Roche) in 2% blocking reagent (Roche) and 10% goat serum (Sigma). The substrate NBT/BCIP (Roche) in 1 M Tris was used for detection.

Transient knock down and over expression

Transient knock down of *tefα* or/and *tefβ* was performed by microinjecting zygotes with 0.3 mM morpholino-modified anti-sense oligonucleotide (Gene Tools) [48], designed to match the *tefα* [$tefα_{(AUG)}$MO: 5′-CGTGATGGAAATAGGCTTCATGTCC-3′] or *tefβ* [$tefβ_{(AUG)}$MO: 5′-CTGAAGACATCTCAGAACGGTT-TCA-3′] initiation of translation regions. In the case of double knock down a final concentration of 0.5 mM was used. No significant difference in mRNA level was observed between mock injected and untreated embryos. As a control the ATG region of *tef* was cloned in frame of *egfp* lacking its endogenous start codon. The chimeric *tef-egfp* mRNA was co-injected with the corresponding morpholino. We observed suppression of EGFP expression for both morpholinos (Figure S2). For transient over expression *tefα* was cloned into pCS2+ and synthesis of capped mRNA was performed with the SP6 mMessage mMachine components

(Ambion). The transcript was column purified (Qiagen) and 100 pg *tefα* mRNA was microinjected into each zygote.

Supporting Information

Table S1 Genes that display light suppressed transcription.
Found at: doi:10.1371/journal.pone.0012542.s001 (0.03 MB DOC)

Table S2 Exon junction crossing Real-Time PCR primers.
Found at: doi:10.1371/journal.pone.0012542.s002 (0.05 MB DOC)

Figure S1 Spectrum of compact fluorescent lamp. (A) Spectrum of the light source that was used for all experiments, showing no emission in the hazardous UV-C (below 280nm), and B (320nm-280nm) class, and minimal emission of least harmful UV-A light (range 400nm-320nm). (B) Experimental setup.
Found at: doi:10.1371/journal.pone.0012542.s003 (1.07 MB TIF)

Figure S2 *tef* knock down control experiment. To assess the capability of the morpholino to knock down its target, the ATG region of *tef* was cloned in front of *gfp* lacking its endogenous start codon, and the *tef-gfp* mRNA was co-injected with the morpholino. (A) $tef\alpha_{ATG}$-*gfp* expression. (B) $tef\alpha_{ATG}$-*gfp* co-injected with corresponding morpholino. (C) $tef\beta_{ATG}$-*gfp* expression. (D) $tef\beta_{ATG}$-*gfp* co-injected with corresponding morpholino.
Found at: doi:10.1371/journal.pone.0012542.s004 (1.74 MB TIF)

Acknowledgments

We are grateful to Carl Neumann and Wilhelmina Baauw for their support. We thank Sabina Radeva for technical assistance, Henry Werner for co-designing and constructing the experimental setup, Jonathon Blake for advice on the computational analysis, and Dan Edvardsson for excellent animal care. We thank Henry Roehl, Florian Maderspacher, and Margot Scheffer for their comments on several versions of this manuscript.

Author Contributions

Conceived and designed the experiments: DG MPSD. Performed the experiments: DG SF TI JS MPSD. Analyzed the data: DG VB MPSD. Contributed reagents/materials/analysis tools: VB MPSD. Wrote the paper: MPSD.

References

1. Sancar A (2004) Photolyase and cryptochrome blue-light photoreceptors. Adv Protein Chem 69: 73–100.
2. Essen LO (2006) Photolyases and cryptochromes: common mechanisms of DNA repair and light-driven signaling? Curr Opin Struct Biol 16: 51–59.
3. Pittendrigh CS (1993) Temporal organization: reflections of a Darwinian clock-watcher. Annu Rev Physiol 55: 16–54.
4. Gehring W, Rosbash M (2003) The coevolution of blue-light photoreception and circadian rhythms. J Mol Evol 57: S286–289.
5. Kang TH, Reardon JT, Kemp M, Sancar A (2009) Circadian oscillation of nucleotide excision repair in mammalian brain. Proc Natl Acad Sci USA 106: 2864–2867.
6. Chen Z, McKnight SL (2007) A conserved DNA damage response pathway responsible for coupling the cell division cycle to the circadian and metabolic cycles. Cell Cycle 6: 2906–2912.
7. Whitmore D, Foulkes NS, Sassone-Corsi P (2000) Light acts directly on organs and cells in culture to set the vertebrate circadian clock. Nature 404: 87–91.
8. Carr AJ, Whitmore D (2005) Imaging of single light-responsive clock cells reveals fluctuating free-running periods. Nat Cell Biol 7: 319–321.
9. Wager-Smith K, Kay SA (2000) Circadian rhythm genetics: From flies to mice to humans. Nat Genet 26: 23–27.
10. Cermakian N, Pando MP, Thompson CL, Pinchak AB, Selby CP, et al. (2002) Light induction of a vertebrate clock gene involves signaling through blue-light receptors and MAP kinases. Curr Biol 12: 844–848.
11. Hirayama J, Miyamura N, Uchida Y, Asaoka Y, Honda R, et al. (2009) Common light signaling pathways controlling DNA repair and circadian clock entrainment in zebrafish. Cell Cycle 8: 2794–801.
12. Hirayama J, Cardone L, Doi M, Sassone-Corsi P (2005) Common pathways in circadian and cell cycle clocks: light-dependent activation of Fos/AP-1 in zebrafish controls CRY-1a and WEE-1. Proc Natl Acad Sci U S A 102: 10194–10199.
13. Whitmarsh AJ, Davis RJ (1996) Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. J Mol Med 10: 589–607.
14. Porterfield VM, Piontkivska H, Mintz, EM (2007) Identification of novel light-induced genes in the suprachiasmatic nucleus. BMC Neurosci 8: 98–107.
15. Gachon F, Olela FF, Schaad O, Descombes P, Schibler U (2006) The circadian PAR-domain basic leucine zipper transcription factors DBP, TEF, and HLF modulate basal and inducible xenobiotic detoxification. Cell Metab 4: 25–36.
16. Haas NB, Cantwell CA, Johnson PF, Burch JB (1995) DNA-binding specificity of the PAR basic leucine zipper protein VBP partially overlaps those of the C/EBP and CREB/ATF families and is influenced by domains that flank the core basic region. Mol Cell Biol 15: 1923–1932.
17. Tamai TK, Vardhanabhuti V, Foulkes NS, Whitmore D (2004) Early embryonic light detection improves survival. Curr Biol 14: R104–105.
18. Dekens MPS, Whitmore D (2008) Autonomous onset of the circadian clock in the zebrafish embryo. EMBO J 27: 2757–2765.
19. Menger GJ, Lu K, Thomas T, Cassone VM, Earnest DJ (2005) Circadian profiling of the transcriptome in immortalized rat SCN cells. Physiol Genomics 21: 370–381.
20. Todo T, Ryo H, Yamamoto K, Toh H, Inui T, et al. (1996) Similarity among the Drosophila (6-4)photolyase, a human photolyase homolog, and the DNA photolyase-blue-light photoreceptor family. Science 272: 109–112.
21. Thresher RJ, Vitaterna MH, Miyamoto Y, Kazantsev A, Hsu DS, et al. (1998) Role of mouse cryptochrome blue-light photoreceptor in circadian photoresponses. Science 282: 1490–1494.
22. Kobayashi Y, Ishikawa T, Hirayama J, Daiyasu H, Kanai S, et al. (2000) Molecular analysis of zebrafish photolyase/cryptochrome family: two types of cryptochromes present in zebrafish. Genes Cells 5: 725–738.
23. Tamai TK, Young LC, Whitmore D (2007) Light signaling to the zebrafish circadian clock by Cryptochrome 1a. Proc Natl Acad Sci USA 104: 14712–14717.
24. Hirayama J, Cho S, Sassone-Corsi P (2007) Circadian control by the reduction/oxidation pathway: catalase represses light-dependent clock gene expression in the zebrafish. Proc Natl Acad Sci USA 104: 15747–15752.
25. Higa LA, Wu M, Ye T, Kobayashi R, Sun H, Zhang H (2006) CUL4-DDB1 ubiquitin ligase interacts with multiple WD40-repeat proteins and regulates histone methylation. Nat Cell Biol 8: 1277–1283.
26. Whitfield ML, Sherlock G, Saldanha AJ, Murray JI, Ball CA, et al. (2002) Identification of genes periodically expressed in the human cell cycle and their expression in tumors. Mol Biol Cell 13: 1977–2000.
27. Dekens MPS, Santoriello C, Vallone D, Grassi G, Whitmore D, et al. (2003) Light regulates the cell cycle in zebrafish. Curr Biol 13: 2051–2057.
28. Bandaru V, Sunkara S, Wallace SS, Bond JP (2002) A novel human DNA glycosylase that removes oxidative DNA damage and is homologous to Escherichia coli endonuclease VIII. DNA Repair 1: 517–529.
29. Tanida H, Tahara E, Mochizuki M, Yamane Y, Ryoji M (2005) Purification, cDNA cloning, and expression profiles of the cyclobutane pyrimidine dimer photolyase of Xenopus laevis. FEBS J 272: 6098–6108.
30. Weissbach H, Etienne F, Hoshi T, Heinemann SH, Lowther WT, et al. (2002) Peptide methionine sulfoxide reductase: structure, mechanism of action, and biological function. Arch Biochem Biophys 397: 172–178.
31. Tew KD (2007) Redox in redux: Emergent roles for glutathione S-transferase P (GSTP) in regulation of cell signaling and S-glutathionylation. Biochem Pharmacol 73: 1257–1269.
32. Carayon P, Portier M, Dussossoy D, Bord A, Petitprêtre G, et al. (1996) Involvement of peripheral benzodiazepine receptors in the protection of hematopoietic cells against oxygen radical damage. Blood 87: 3170–3178.
33. Bedo G, Vargas M, Ferreiro MJ, Chalar C, Agrati D (2005) Characterization of hypoxia induced gene 1: expression during rat central nervous system maturation and evidence of antisense RNA expression. Int J Dev Biol 49: 431–436.
34. Wandinger SK, Richter K, Buchner J (2008) The Hsp90 chaperone machinery. J Biol Chem 283: 18473–18477.
35. Delaunay F, Thisse C, Thisse B, Laudet V (2003) Differential regulation of Period 2 and Period 3 expression during development of the zebrafish circadian clock. Gene Expr Patterns 3: 319–324.
36. Ziv L, Levkovitz S, Toyama R, Falcon J, Gothilf Y (2005) Functional development of the zebrafish pineal gland: light-induced expression of period2 is required for onset of the circadian clock. J Neuroendocrinol 17: 314–320.
37. Cheng HY, Papp JW, Varlamova O, Dziema H, Russell B, et al. (2007) microRNA modulation of circadian-clock period and entrainment. Neuron 54: 813–829.
38. Fonjallaz P, Ossipow V, Wanner G, Schibler U (1996) The two PAR leucine zipper proteins, TEF and DBP, display similar circadian and tissue-specific

expression, but have different target promoter preferences. EMBO J 15: 351–362.
39. Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, et al. (2002) The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell 110: 251–260.
40. Xu X, Liu L, Wong KC, Ge R (1998) Cloning and characterization of two isoforms of the zebrafish thyrotroph embryonic factor (tef alpha and tef beta). Biochim Biophys Acta 1395: 13–20.
41. Vatine G, Vallone D, Appelbaum L, Mracek P, Ben-Moshe Z, et al. (2009) Light directs zebrafish period2 expression via conserved D and E boxes. PLoS Biol 7: e1000223.
42. Falvey E, Fleury-Olela F, Schibler U (1995) The rat hepatic leukemia factor (HLF) gene encodes two transcriptional activators with distinct circadian rhythms, tissue distributions and target preferences. EMBO J 14: 4307–4317.
43. Gachon F, Fonjallaz P, Damiola F, Gos P, Kodama T, et al. (2004) The loss of circadian PAR bZip transcription factors results in epilepsy. Genes Dev 18: 1397–1412.
44. Drolet DW, Scully KM, Simmons DM, Wegner M, Chu KT, et al. (1991) TEF, a transcription factor expressed specifically in the anterior pituitary during embryogenesis, defines a new class of leucine zipper proteins. Genes Dev 5: 1739–1753.
45. Ishikawa T, Hirayama J, Kobayashi Y, Todo T (2002) Zebrafish CRY represses transcription mediated by CLOCK-BMAL heterodimer without inhibiting its binding to DNA. Genes Cells 7: 1073–1086.
46. Mullins MC, Hammerschmidt M, Haffter P, Nüsslein-Volhard C (1994) Large-scale mutagenesis in the zebrafish: in search of genes controlling development in a vertebrate. Curr Biol 4: 189–202.
47. Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30: e36.
48. Nasevicius A, Ekker SC (2000) Effective targeted gene 'knockdown' in zebrafish. Nat Genet 26: 216–220.

1.2 Discussion

In our study, we identified 19 transcripts unregulated in light, whose transcription is dependent on the PAR bZIP transcription factor Tef α and a set of 12 transcripts downregulated in the presence of light. Surprisingly, an overwhelming majority of transcripts were involved in DNA damage repair and stress response. The study was performed in several stages, with a first pilot study carried out at 75% epiboly rather than 90% used in later studies. Downregulated transcripts were only observed in early stages, indicating that light-mediated down-regulation may be stage-specific. Weger and colleagues investigated the light responsive transcriptome of zebrafish PAC2 cells, *in vitro* cultured heart and in larvae. The authors identified 117 light regulated genes of which 90 were upregulated by light and 27 were downregulated. Similarly to our study, Weger and colleagues observed numerous transcripts involved in DNA repair as well as other functions such as retinal light reception and metabolism. In our study, the downregulation of the downregulated transcripts could not be confirmed by qPCR and therefore was omitted from the enclosed manuscript(described in supplementary materials Table2). Curiously, Weger and colleagues observed downregulation of a similar set of genes, further indicating that light-repressed genes may be stage-specific. Furthermore, as thyrotroph embryonic factor is know to function as a homo- or heterodimer with DBP or HLF or E4BP4, we tested whether the expression of the two DBP, HLF and E4BP4 genes cycled in early zebrafish larvae, although these transcripts were not identified as upregulated in the microarray assay. Indeed, we did not observed cyclic expression of the HLF, DBP and E4BP4 transcripts analyzed by qPCR on cDNA obtained from whole larvae. Weger and colleagues however, identified DBP in their microarray screen in the "cell set" as downregulated, indicating that DBP may be expressed in a light-dependent or cyclic fashion only later in zebrafish development [96]. Recently, Ben Moshe and colleagues identified two hlf, two dbp and six e4bp4 genes in the zebrafish (Zv7), that were successfully cloned and their circadian expression was analyzed by WMISH[97]. We were not able to identify as many homologs during our bioinformatic analysis. This discrepancy may be due to the fact that we utilized an early zebrafish genome assembly (Zv6)[97]. Ben Moshe and colleagues observed a peak in tef β expression beginning ZT18 with a maximum at ZT22 in the pineal of late zebrafish larva. In our study we evaluated gene expression in the whole larva rather than the pineal and observed an upregulation in the dark, with a peak at ZT15 from the 4 time points measured. Using WMISH, we also observed tef expression in the pineal (Fig.2 of the enclosed manuscript). Ben Moshe observed light-mediated expression of both hlf1 and hlf2 in the pineal. We did not observe any change in expression in the whole larvae. Ben Moshe also demonstrates strong expression of hlf genes in the retina in a non-light dependent or circadian fashion. As the pineal is quite small, it may be that the differences in gene expression observed by Ben Moshe in the pineal could not be observed due to background from other tissues expressing these proteins in a non-light dependent manner. As Tef can form homodimers with itself or heterodimer with other PAR proteins, based on Ben Moshe's data, one can postulate that in the pineal Tef could dimerise with Hlf2 , Dpb1 and Dbp2 could be inhibited by E4BP4-6 [97], indicating a far more complex regulatory system in later zebrafish development.

Weger and colleagues furthermore, identified the promoter elements necessary for

light dependent transcription and demonstrated that these motifs were indeed sufficient to drive a light-mediated bioluminescence reporter gene expression [96]. Our promoter analysis was not as in depth as that of Weger and colleagues. However, in both studies Morpholino-modified antisense oligonucleotides were used to determine whether tef expression affected the expression of the other upregulated genes. Weger and colleagues observed that tef α and β knockdowns attenuated expression of per2 and lonrf (1of2), whereas tef α knockdown affected cry5 expression and ddb2 and cry1a were not affected by either tef knockdown. In our study, we observed global downregulation by tef α and β, although tef α had a considerably stronger effect. The differences observed may be due to the methods used. Weger and colleagues collected their observations in cell culture, whereas we used young larvae at 9hpf. Tef α and β may indeed carry out different functions at later stages in the development of zebrafish [96].

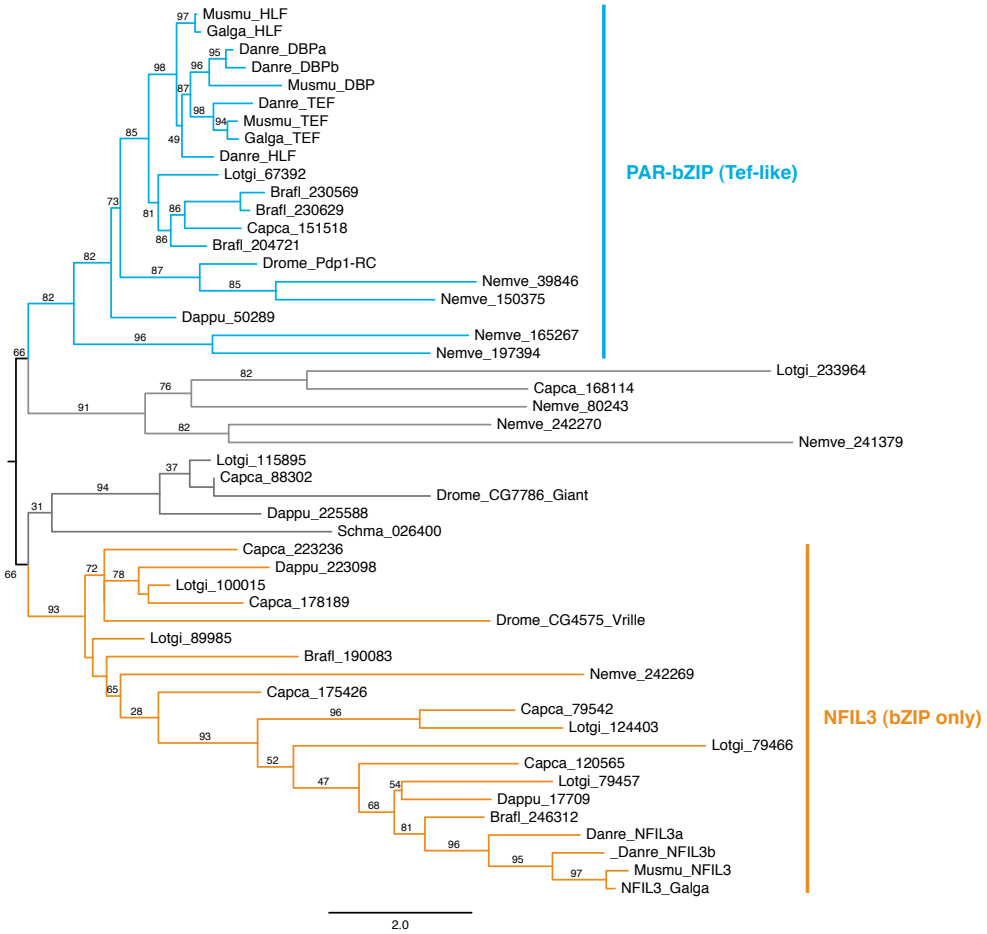
Our work revealed that the non-UV radiating light induces DNA repair and stress gene expression. Similar results obtained using different methods by Weger and colleagues further corroborate this hypothesis. We postulated that Tef could play a pivotal role in coupling the circadian clock to light-induced repair in young zebrafish. Results by Weger indicate that the complex timing of expression of the different PAR partners may hide a far more complex regulatory mechanism than previously envisaged.

These curious observations are also interesting in respect to the evolutionary origin of such systems. Blast searches for PAR bZIP homologs in NCBI and using TreeFam yielded a surprising diversity of homologs.

Phylogenetic analysis of PAR bZIP proteins and the bZIP NFIL3 family revealed the presence of three large groups: one grip containing the "Tef-like" vertebrate proteins including TEF, HLF and DBP as well as sequences from *Branchiostoma*, *Capitella* and *Drosophila* PDP1, a second group containing the vertebrate NFIL3 (group containing no PAR domain but a homologous bZIP domain) and *Branchiostoma*, *Capitella* and *Lottia* sequences as well as the*Drosophila* VRILLE, and a third group containing invertebrate-only sequences (Fig. 1.1).

In vertebrates, PAR bZIP transcription factors function as dimers, either homo or heterodimers of TEF, HLF and DBP. These proteins have also been described to be able to also interact with other bZIP transcription factors such as CREBP. Phylogenetic analysis revealed that the three vertebrate PAR bZIP proteins (TEF, HLF and DBP) form a single group, indicating duplication events specific to the vertebrate lineage. However, there is also indication of the presence of at least one "true" PAR bZIP in the ancestor of bilaterians, as *Branchiostoma*, *Capitella* and *Nematostella* PAR bZIP domain containing sequences group with the vertebrate TEF, HLF and DBP cluster. Moreover, the third group containing invertebrate only sequences curiously contains the *Drosophila* giant protein. The domains observed in this group were not uncovered in vertebrates. The two large groups containing Tef-like and bZIP proteins, unlike the third one, enjoy strong support and contain vertebrate and invertebrate sequences. Thus it is likely that at least one NFIL-like and one Tef-like gene was present in Urbilateria. There appear to have been numerous duplications in *Nematostella*. Due to the conservation of circadian-like function of PAR bZIP proteins in *Drosophila*, it is clear that this gene family deserves more attention. Whether in vertebrates or *Drosophila* PAR bZIP proteins have been shown to be important in circadian and light induced gene expression regulation. Their function in lophotrochozoa is however not yet been inves-

Figure 1.1: Phylogenetic tree of PAR bZIP transcription factors Sequence alignment and phylogenetic trees were made using PhyML using a Lg $+\gamma$ CAT model. Support are SH LRT p-values. Phylogenetic analysis was performed in collaboration with Dr. Ferdinand Marletaz (Arendt lab, EMBL, Heidelberg)



tigated, despite clear indication from phylogenetic analysis that this gene family was present in Urbilateria.

Chapter 2

Dissociation of *Platynereis* into individual cells

2.1 Introduction

Like all multicellular organisms *Platynereis dumerilii* are made up of their individual cells, which interact with each other and their environment. An *in vitro* study of *Platynereis* cell types has the advantage of providing the possibility to encompass the multitude of factors that make up the complexity of a living system.

The first step in the analysis of individual "single" cell analysis is obviously to be able to identify and to isolate the cells of interest amidst the mixture of contaminating cells. The process of isolation involves the disaggregation of whole organisms, organs or tissues into their cellular components with the least possible damage to their integrity. Indeed, harsh treatment with chemicals, changes in temperature and salinity can affect morphology, behavior and gene expression and much care should thus be taken to avoid affecting the cells that are to be studied. Numerous methods have been proposed for sorting of cells of interest, based on various properties such as size, morphology, density and gene expression. Although, flow cytometry has been widely implemented as a method for sorting cells in suspension, using this method cells can only be defined by a single character in respect to which they may be identical although significant heterogeneity may be observed in other respects [98]. Furthermore, flow cytometry based on fluorescent reporter gene expression implies *a priori* knowledge of gene expression in cells of interest and capability to perform efficient transgenesis with desired cells. Before the advent of flow cytometry, cells were routinely sorted by size and morphology using different size mesh diameters or density gradient centrifugation. Numerous studies have also taken advantage of advances in some form of microdissection (reviewed in [99, 100, 98]). However, such methods are often labor-intensive and time consuming, resulting in degraded RNAs hindering subsequent transcript analysis.

Furthermore, tools to investigate cell behavior are necessary. My goal has been to establish such tools and provide a means to characterize and culture cells on the short term. Establishment of tools to dissociate *Platynereis* is described in this chapter. The

characterization of the resulting disaggregated cells is discussed in Chapter 3. Tests performed on the maintaining alive the cells separated from the whole organism in *ex vivo* conditions providing for their nutritive and environmental needs are dealt with in Chapter 4.

Dissociation of cells for primary culture from organisms other than *Platynereis* is usually achieved by a combination of dissection, mild proteolytic treatment of basement membranes and other connective tissues, and physical separation of cells [101]. Methods for efficient dissociation of numerous standard model organisms have been established during the last few decades. However, there are no standard protocols for obtaining cells from understudied model systems, and particularly, from marine invertebrates. A multitude of methods have been successively proposed on organisms of the same phylum, of the same species, for the obtention of the even same cell type and by the same authors. In all cases, the dissociation process consists of breaking down the integrity of the tissues and of incapacitating the molecules exerting an adhesive action between cells. The latter may vary from organism to organism.

Poriferans are known to dissociate "spontaneously" by simple incubation in a solution lacking calcium necessary for adherens junctions. Gentle agitation and mechanical pressure were shown to increase the yield of dissociation [102]. Enzymatic treatment has resulted in the destruction of cells, and a lower yield of individual cells with a smaller cell diversity. Similarly, incubation in sterile seawater solutions or mild mechanical dissociation has been shown to be sufficient for dissociation. Schmid and colleagues described the dissociation of the cnidarian *Podocoryne carnea* by gentle heating in a sterile seawater solution [103]. Gates and Muscatine observed similar results [104]. Chemical dissociation of cnidarians yielded smaller numbers of viable cells as compared to mechanical dissociation, using Trypsin, however Collagenase and Pronase as well as EDTA treatment were seen as less harmful to the cells. The size distribution of viable cells obtained after the chemical dissociation did not differ from that of mechanical dissociation (5 to 25 microns). Khalesi however observed that treatment with Trypsin-EDTA yielded the highest viability of cells derived from *Sinularia flexibilis* [105]. This discrepancy is representative of most marine animal phyla, in which the success rate of dissociation differs not only from cell type but from author to author.

Mechanical dissociation appears to be sufficient in loosely bound tissues and organisms such as poriferans or cnidarians. Chemical treatment is necessary for tissues and organisms bound more tightly. A variety of enzymatic cocktails have been used, generally including specific proteases such as Trypsin and Collagenase.

- Trypsin is a pancreatic serine protease with a specificity for peptide bonds involving the carboxyl group of the basic amino acids, arginine and lysine and is one of the most highly specific proteases known, although it also exhibits some esterase and amidase activity [101]. Using specific proteases involves running the risk of specifically digesting transmembrane proteins that may be important for cell viability.

- Bacterial collagenase is a crude complex containing a collagenase more accurately referred to as clostridiopeptidase A which is a protease with a specificity for the X-Gly bond in the sequence Pro-X-Gly-Pro, where X is most frequently a neutral amino acid. Such sequences are often found in collagen, but only rarely

in other proteins. Collagenase treatment has been successful in organisms and tissues containing collagen such as Molluscs and questionably in oysters and where the presence of collagen has not been described [106, 101].

- Elastase is a serine protease with a specificity for peptide bonds adjacent to neutral amino acids. It is frequently employed to dissociate tissues containing extensive intercellular fiber networks [101].
- Pronase, Proteinase K and Papain are large spectrum non-specific proteases that consist of a cocktail of different proteolytic enzymes [101].
- Although some cells naturally aggregate such as cells derived from poriferans and cnidarians, cells derived from other sources can be treated with substances preventing aggregation. Dispase has been demonstrated to help prevent cell aggregation. As DNA liberated from lysed cells may favor aggregation, DNase I or Hyaluronidase treatment has been advised in numerous cases (Fig. 2.1).

Early *Platynereis* larvae are surrounded by a tough fertilization membrane, yet the blastomeres are loosely attached. Whereas older larvae and adult tissues are more tightly bound. Different dissociation conditions were tested for the different stages tested. The limitations of working with *Platynereis* include the fact that the size of larvae and adults makes dissection challenging. Furthermore, source material is scarce. Therefore, efforts were primarily concentrated on determining which dissociation conditions could produce the highest yield in terms of viable cells and could disaggregate tissues into individual cells most efficiently, from low amounts of living organism. The criteria utilized to achieve this goal were:

1. amount of cells obtained
2. the relative amount of individual cells vs. cells in aggregates
3. survival of cells (absence of osmotic shock, cell death ...)

2.2 Results

2.2.1 Dissociation of *Platynereis* larvae (24hpf-28hpf)

Qualitative dissociation method screen

No publications describing the dissociation of *Platynereis dumerilii* or any related marine polychaete species was uncovered in literature, and an overview of literature concerning the methods employed for the dissociation of other marine invertebrates yielded no consensus either between organisms, tissues or yield (Fig. 2.1), a preliminary small-scale qualitative dissociation screen was performed using six proteolytic solutions in order to determine their relative efficiencies and time necessary for dissociation. Larvae (aged 24-48hpf) were treated with enzymes at concentrations of 5%, 1% and 0,075-0.01% and dissociation efficiency was determined over time. Both commercially available powdered solutions and liquid solutions were used and resuspended

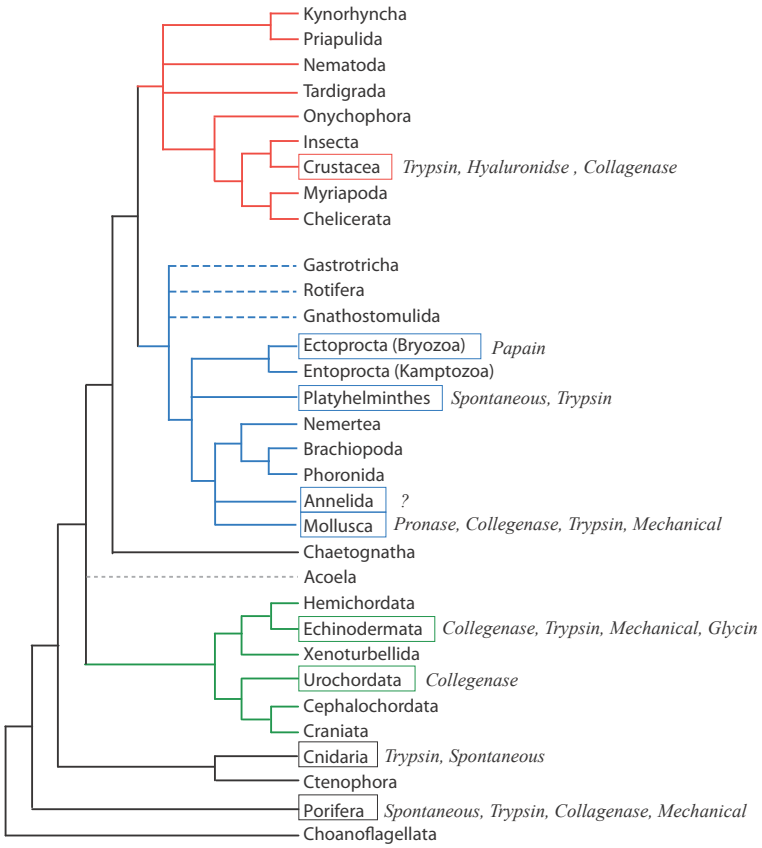


Figure 2.1: Different methods used for dissociating different marine invertebrates. Tree based on [107].Data for Porifera from [102, 108, 109, 110], for Cnidarians [111, 112, 103, 105, 105], for Crustaceans [113, 114, 115, 116, 106, 117, 118, 119, 120, 121, 122, 123], for Molluscs [124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 122]

in Sterile Filtered Natural Seawater (SFNSW) and Calcium-Magnesium Free Sterile Filtered Seawater (SFCMFSW). As cells were derived from a marine organism, care was taken to avoid provoking an osmotic shock. Assays were performed on sets of 100 to 300 larvae in volumes of 200 to 500 μl of final solution. All attempts using Gibco Trypsin-EDTA solution proved unsuccessful and resulted in osmotic shock. Dilutions to appropriate concentrations of SFCMFSW resulted in amounts of enzyme that did not have an effect on the dissociation of *Platynereis* cells after 4.5 hours.

Trypsin Trypsin from powdered solutions (Roche and Promega) could be resuspended directly in SFNSW or SFCMFSW, avoiding osmotic shock. Advance preparation of Trypsin stock solution in DTT as advised by manufacturer was abandoned, as high levels of DTT in the small volumes (200 μl) used for testing resulted in rapid larval death. A viscous substance could be observed flowing out of larvae and movements and swimming behavior ceased. At low concentrations of Trypsin cells were dissociated, however they appeared damaged (Fig. 2.2A-C and Table 2.2.1). Numerous cells appeared larger than expected from the stages dissociated, indicating swelling due to osmosis (Fig. 2.2 A-B and Table 2.2.1 and Table 2.2.1). Many of such cells were observed to burst (2.2C and Table 2.2.1). Incompletely dissociated cell aggregates were not frequently observed. Treatment of larvae with high concentrations of Trypsin (0.075%, 1% and 5%) yielded fewer individual cells upon observation (Fig. 2.2D-F and Table 2.2.1). Large aggregates of cells resulting from incomplete dissociation were frequently observed. Cells in these aggregates did not appear to be damaged, indicating a protective effect of large cell clusters on cell integrity. Large swollen single cells were also observed. Due to its high sequence specificity, Trypsin was suspected to cleave a crucial transmembrane protein present on *Platynereis* cells and deemed unfit for its dissociation. Presence of aggregates at what appeared to be high frequencies, was assumed to result from the death of all individual cells after treatment, leaving mainly the cells that were protected by clusters of surrounding cells. Furthermore, Trypsin at low concentrations (0.3%) has been previously shown to induce oocyte maturation of the polychaete *Chaetopterus* in Artificial Seawater (ASW), demonstrating that an important substrate of Trypsin may be expressed in early polychaete eggs [136].

Collagenase Treatment with collagenase (Fig. 2.2 G-I and Table 2.2.1) was observed as much milder on *Platynereis* cells than Trypsin. Numerous aggregates were observed and few individual cells were recuperated. Cell morphology did not appear affected. Collagenase treatment at all the concentrations tested did not appear to display sufficient proteolytic activity for full dissociation. These results indicates that collagen and other protein carrying this sequence is present but not abundant in *Platynereis* larvae. Presence of collagen has been described in polychaete cuticles [137], however the lack of success in dissociation of *Platynereis* larvae is in line with the low abundance or the absence of collagen fibrils in young polychaete larvae described previously [138, 139, 140, 141].

Proteinase K and Trypsin Treatment with a combination of highly concentrated Proteinase K and Trypsin (Fig. 2.2 J-L and Table 2.2.1) yielded numerous individ-

ual cells. Many of these appeared damaged, swelled and burst. Furthermore, unlike treatment with Trypsin alone and collagenase, large aggregates surrounded by a visible membrane-like structure were observed. They contained a cell carrying a lipid droplet were observed (Fig. 2.2 L and Table 2.2.1). The membrane-like structure was assumed to correspond to the remaining chorion of the larva. Cell clusters were distinctively large, however smaller than whole larvae of the corresponding stage, thus demonstrating that some cells had either been expelled from the larva or had died. As Trypsin was seen in previous tests to not be efficient at the disaggregation of larvae, the effect was considered to be specific to Proteinase K. At low concentrations of Proteinase K and Trypsin (Fig. 2.2 M-O and Table 2.2.1) few aggregates were visible. Numerous cells undergoing an osmotic shock were observed. In combination with the results of treatment at high concentrations, a conclusion similar to that concerning treatment with Trypsin was made. At high concentrations all individual cells were destroyed leaving a majority of incompletely dissociated cell clusters that offered protection. It can also be hypothesized that the extracellular matrix (ECM) between cells was not efficiently affected by the treatment, indicating a lack of substrate for Proteinase K in the *Platynereis* ECM.

Proteinase K alone Treatment with Proteinase K alone confirmed that the appearance of the membrane-like structure surrounding incompletely dissociated cell aggregates was specific to Proteinase K and not Trypsin (Fig. 2.2 P-S and Table 2.2.1). Individual cells were less affected by treatment with Proteinase K than by Trypsin alone or Trypsin in combination with Proteinase K. Digestion with Proteinase K is routine in cuticle and chorion permeabilization in polychaete research. Thus, although, Proteinase K may be efficient at destroying the chorion of larvae, it may also have a deleterious effect upon cell survival after longer incubation periods.

Pronase Treatment with Pronase (Fig. 2.2 S-U and Table 2.2.1) at high concentrations, yielded numerous individual cells, most of which did not appear to be damaged and did not swell or burst.

Pronase and Collagenase/Dispase Digestion with Pronase in combination with Collagenase/Dispase solution (Fig. 2.2V-X and Table 2.2.1) yielded numerous individual cells, cell aggregates and several large cells with non-circular morphologies. NB: preservation of rare differentiated cells with non-circular morphologies was rarely observed after dissociation with any of the other enzymes tested. As collagenase was previously observed to be inefficient at dissociation into single cells, the effect was attributed primarily to the effect of Pronase and the presence of Dispase. Pronase is commonly used in zebrafish for dechorionation of young embryos. Dispase is a bacterial enzyme produced by *Bacillus polymyxa* that hydrolyzes N-terminal peptide bonds of non-polar amino acid residues and is classified as an amino-endopeptidase. Its mild proteolytic action makes the enzyme especially useful for the isolation of primary and secondary (subcultivation) cells since it maintains cell membrane integrity. In comparison, mechanical dounce disaggregation and filtering of cells through cell sorters (Fig. 2.2 Y and Table 2.2.1) yielded a viscous solution even after several washes

with few cells. The latter died rapidly during observation. It was therefore concluded that treatment with Pronase and Dispase was the most efficient method to dissociate *Platynereis* larvae into individual cells.

Dispase vs DNase I Dispase was found to be interchangeable with DNAse I. Treatment with DNase I at 0,01% yielded similar results. Treatment with Papain (not shown) yielded similar results to those observed with Pronase and Proteinase K.

Other enzymes tested Treatment with Elastase and Lysozyme(not shown) had no effect on larvae for up to 4 hours observation. Larvae continued to swim and no sign of damage was observed. Lysozyme is not a conventional enzyme used for cell dissociation and was tested because of its capacity to damage bacterial cell walls. As numerous bacteria are present in *Platynereis* culture conditions, lysozyme treatment was performed in the hope to remove bacterial contamination. However, bacterial contamination observed in all samples was also present in the lysozyme treated sample (Table 2.2.1).

Pipetting the solution up and down with a glass pasteur pipette or pulled capillary at 15 minute intervals did not appear to have a visible effect on the dissociation. Incubation on an embryo shaker, functioning at 500-900rpm increased yield. This was not quantified. However, all experiments described below refer to samples processed on a shaker or rotating table at speeds above 500 rpm. Incubation in of each solution at 37 °C appeared to be deleterious towards the cells.

Figure 2.2 *(following page)*: Qualitative evaluation of dissociation conditions with different enzymes. Low concentrations of Trypsin (A-C) yielded cells that suffer from osmotic shock (white arrows) (A-B), growing in size and then bursting, numerous aggregates (black arrows) were observed (C). After incubation in high concentrations of Trypsin (D-F) (0,75% and higher) few individual cells were observed. Most cells were in aggregates. Several large cells undergoing an osmotic shock were visible. Treatment with Collagenase (G-I) at all concentrations tested yielded cell aggregates and few individual cells. Treatment with over 0,075% Proteinase K and Trypsin (J-L) yielded more individual cells which maintained their morphology (J), some of which were undergoing an osmotic shock and large aggregates surrounded by a large membrane containing a lipid droplet(L). After incubation with low (0.05% and lower) concentrations of Proteinase K and Trypsin (M-O) few aggregates were observed. Many cells were shrinking or bursting from osmotic shock. Treatment with Proteinase K alone (P-R) yielded many large aggregates surrounded by a membrane composed of cells that did not appear to be affected by osmotic shock. Treatment with Pronase at all concentrations yielded aggregates and at high concentrations (over 0,075%) individual cells were observed. They did not appear affected by osmotic shock (S-U). Treatment with Pronase and Collagenase/Dispase solution at all concentrations tested yielded numerous individual cells and numerous cells with non-circular morphologies were preserved(V-X). Mechanical disaggregation did not successfully separate cells, yielding large aggregates attached to each other through a viscous substance(Y).

Trypsin
Collagenase
Proteinase K & Trypsin
Proteinase K
Pronase
Pronase & Collagenase/ Dispase
Mechanical
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y
Low concentrations:
- osmotic shock
- numerous aggregates
High concentrations
- few individual cells
- most cells in aggregates
- cells undergoing osmotic shock
- aggregates
- few individual cells
High concentrations:
-osmotic shock
-large aggregaetes
Low concentration:
-membrane bound large aggregates
- no osmotic shock
- large aggregates
- no osmotic shock
-numerous individual cells
-non-circular morphology preserved
- individual cells
- preserved morphology
- aggregates
- aggregates in viscous substance

Table 2.1: Summary of qualitative evaluation of dissociation conditions of *Platynereis* larvae

Method tested	Individual cell disaggregation	Yield or survival
Trypsin	+++	+
Collagenase	+	+++
Collagenase/Dispase	++	++
Proteinase K	+++	+++
Pronase	+++	++++
Pronase & Collagenase/Dispase	++++	+++
Papain	+++	++
Elastase	-	-
Lysozyme	-	-
Mechanical dissociation	-	++

Quantitative evaluation of cell viability of the enzymatic dissociation of *Platynereis* larvae

The qualitative comparison of enzymatic and mechanical treatments to obtain dissociated cells from *Platynereis* showed Pronase, Papain and Proteinase K performed better than Collagenase, Trypsin, Lysozyme, Elastase and mechanical dissociation in terms of the amount of cells yielded, the preservation of cell morphology and the capacity to yield individual cells. In order to confirm these observations in a quantitative manner, several quantitative assays were tested.

1. fluorescein diacetate (FDA) de-esterification [142]
2. 3-4,5, dimethylthiazol-2-yl-2,5-diphenyl tetrazolium (MTT)reduction [143]
3. trypan blue exclusion test [144, 145]
4. neutral red (NR) incorporation [146]
5. cell number evaluated using a hemocytometer

Furthermore, no description of these tests for *Platynereis* cells was obtained in literature, although they are routinely used in other organisms. To ascertain that *Platynereis* cells are capable of FDA de-esterification, MTT-reduction and NR incorporation and TP exclusion, a pilot assay was performed. The results observed indeed confirmed that *Platynereis* cells were capable of FDA de-esterifcation of MTT-reduction. Pilot tests with NR and TP, indeed confirmed that *Platynereis* incorporated NR into putatively lysozomal structures and living cells excluded TP. NR incorporation been measured using a luminometer in order to measure viability of cells in shrimp digestive acini, however, in my hands NR incorporation was not replicable. Considerable NR in the medium remained in the wells and biased any spectrophotometric reading. Therefore, NR incorporation and TP exclusion were evaluated manually (Fig. 2.5). Cells were therefore counted using a hemocytometer. Furthermore, as the MTT and FDA assay

yield information concerning the viability of any living tissue present in the 96-well plate well, non-dissociated larvae and non-dissociated portions of larvae were removed using a 75 micron nylon mesh. Thus any cell aggregate or non-dissociated larvae were retained on the mesh whereas any cell aggregates or individual cells smaller than 75 microns flowed through and were collected in an eppendorf. The flowthrough of this procedure corresponds to the sample measured in all assays.

The MTT-reduction assay (CellTiter 96-Promega) takes place over a period of 2 hours during which metabolically active cells cleave the reagent to a dark colored formazan product whose concentration is measured using a 96-well plate reader. The fluorescence based assay works faster than the MTT-reduction assay. The fluorescence incorporated into cells can be measured immediately after dissociation and washing. Therefore, the comparison between the two tests yields information about the amount of viable cells immediately after dissociation and the amount of cells still metabolically active several hours after dissociation. Cell counts were performed using a hemocytometer. Neutral red incorporation measures the number of living cells, whereas Trypan blue measures the number of dead cells, rather than the viability. Total cell counts were also carried out. In order to confirm the results of the qualitative assay, the same enzymatic treatments were tested in the same conditions (5%, 1% and 0,075-0.01% enzyme in 200 μl reaction of 100 larvae). Two replicates were measured for each condition tested.

Tests were performed on the promising candidates from the qualitative evaluation: Pronase, Proteinase K, and Papain.

Papain Cells obtained by treatment with Papain over 3 hours demonstrated higher MTT-reduction than cells obtained after 1 hour. The trend was dependent on the concentration of enzyme with higher concentrations of enzyme performing better (Fig. 2.4A). Immediate cell viability as measured with FDA demonstrated higher viability of cells obtained after 1 hour treatment than 3 hours (Fig. 2.4B). This discrepancy is not due to the number of cells obtained as the yield remained relatively stable (Fig. 2.4D). Neutral red incorporation demonstrated the opposite trend to that observed with the MTT-reduction assay in respect to concentration dependency, namely higher amounts of cells capable of incorporating NR with lower concentrations of enzyme used and a similar tendency in terms of incubation time, with cells obtained after 1 hour treatment performing better than cells obtained after 3 hours treatment. This indicates, that the metabolic processes of *Platynereis* is modified by such conditions. Papain treatment affected NR incorporation in a different fashion than mitochondrial dehydrogenase activity. As NR incorporation is dependent on active transport of molecules, perhaps key pumps were affected by the enzymatic treatment. Quantitative evaluation of cell dissociation with Pronase demonstrated a different trend. The MTT-reduction assay indicated similar metabolic activity at all concentrations and time periods tested, with slightly better results at higher concentrations (1%-0.05%) after 3 hours (Fig. 2.4E). The FDA-based assay did not show a clearly interpretable trend that could be easily compared to that of the MTT assay (2.4F). Slightly better results were observed in cells that underwent treatment at 5% and 0.75% Pronase (Fig. 2.4F). NR incorporation and total cell count demonstrated a similar trend (Fig. 2.4G-H). A concentration

dependent increase in NR incorporation and cell number could be observed at both time points tested. Lower enzyme concentrations yielded better results than higher concentrations.

Proteinase K Proteinase K treatment showed a similar trend. Lower enzyme concentrations yielded higher MTT-reduction values and FDA fluorescence (Fig. 2.4I-J). Total cell number yields showed higher yields at extreme high and extreme low concentrations (Fig. 2.4L). The NR incorporation assay failed as in the samples designated for this purpose, all cells died unexpectedly (Fig. 2.4K). Trypan blue exclusion did not contribute to the analysis, as it much resembled the trend observed in total cell number and NR incorporation. Increasing amounts of cells were observed to die with decreasing concentrations of Papain. This is in contradiction with the results of the MTT assay which would indicate higher viability at these concentrations. Perhaps, a transmembrane protein or pump necessary for Trypan blue exclusion or incorporation may have been affected by the enzymatic treatment.

Pronase MTT-reduction assay values overall were higher for treatments with Pronase (Fig. 2.3A), whereas overall FDA values were highest for Papain treatments (Fig. 2.3B). PK values for all assays were the lowest. NR values were comparable for all treatments undertaken (Fig. 2.3C). Absolute cell counts were highest for Pronase treatments (Fig. 2.3D).

The values obtained from these assays were performed in duplicates. The scarcity of starting material rendered higher replicate number difficult. The readings obtained from the replicates are not identical and may appear non-replicable. One must remember the method utilized to obtain this data. The solutions containing the dissociation solution and the putatively dissociated larvae were filtered through a nylon mesh and replated into a 96-well plate. Considerable loss may have taken place during this step.

These results were observed for sets of one hundred larvae incubated in relatively low volumes (200μl). When dissociating larger sets of larvae such as whole batches (c.1000 larvae) concentrations and volumes need to be adapted to the dilution factor. Based on the above observations, Papain and Pronase were deemed most suitable for dissociation of *Platynereis* cells. Quantitative measurements obtained with Papain appeared irreplicable. Furthermore, papain is not readily dissolved in seawater and requires an additional resuspension step in DTT whilst heating step, judged as impractical in comparison to Pronase which is readily dissolved in seawater. For this reason in all subsequent assay, Pronase was utilized at concentrations of 0.075 to 0.025%. As cell yield varied between the two time points examined, an additional assay was performed in order to determine the time necessary to obtain the maximum amount of cells from 20 larvae of a time course of several hours (Fig. 2.2.2). This experiment was carried out on young embryos and demonstrated that longer incubation times were necessary for maximum yield. Dissociation experiments performed on whole batches (approx. 1000-1500 larvae) required extended time intervals ranging up to four to five depending on batch size. Dissociation quality on whole batches was evaluated by the cloudiness of the solution. When cells begin to lyze the lipid droplets that they contain are liberated and provide a cloudy appearance to the dissociation solution.

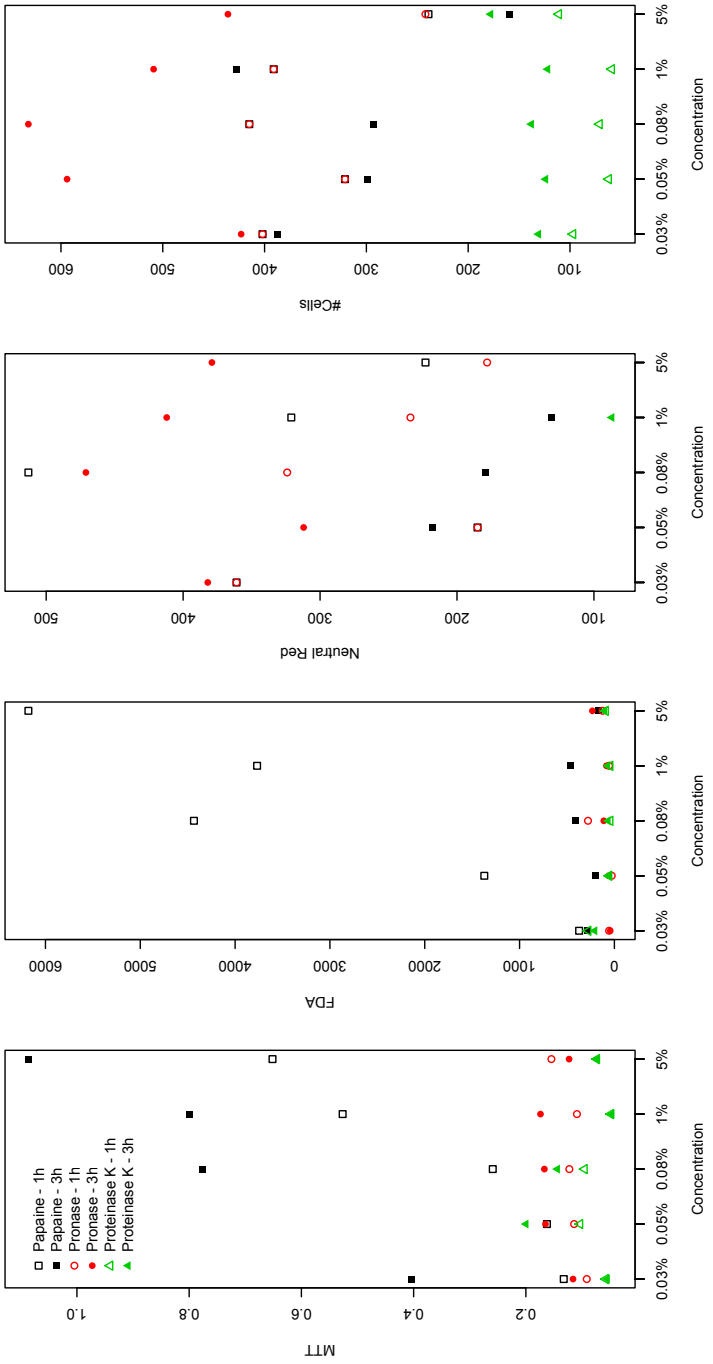


Figure 2.3: Comparison of different concentrations of dissociation enzymes using 4 assays from left to right: MTT-reduction assay, FDA-esterification assay, Neutral Red incorporation assay and the total amounts of cells counted in 200 μl using a hemocytometer.

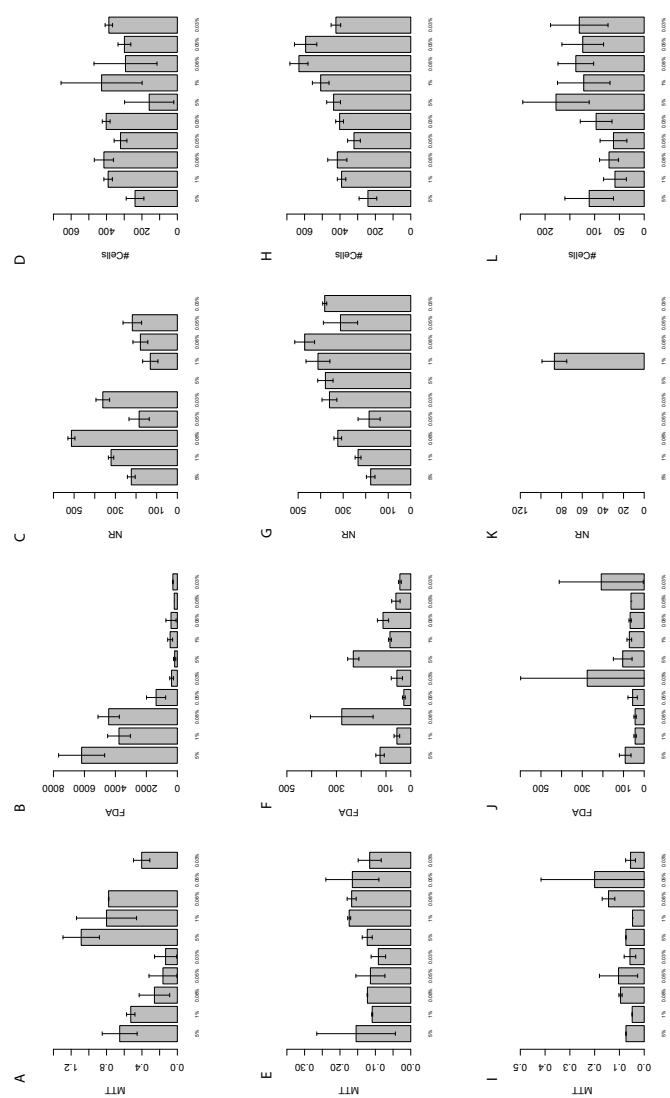


Figure 2.4: Quantitative measurement of cells obtained by treatment with Papain(A-D), Pronase(E-H) and (Proteinase K)(I-L) using the MTT-reduction assay (A, E and I), FDA-esterification assay (B,F and J), NR incorporation (C, G and K) and total amounts of cells counted in 200 μl using a hemocytometer (D, H and L). (NB: absence of bars in A 0.05%, C 5% and all concentrations aside from 1% in K do not correspond to low values but experiments that did not yield possible quantification and could not be replicated due to the technical difficulties discussed in the text)

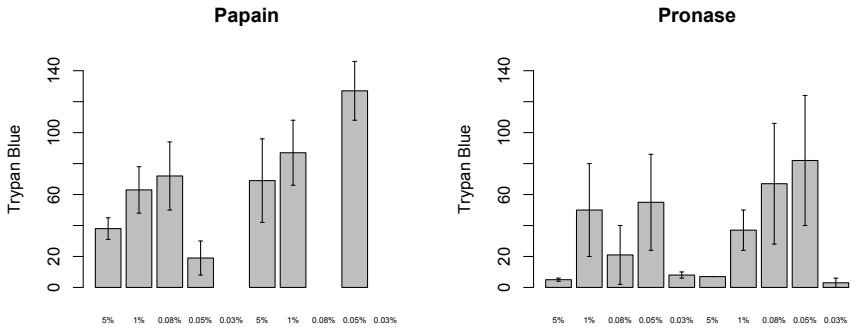


Figure 2.5: Trypan blue exclusion assay after treatment with Pronase and Papain

Dissociation of adult *Platynereis dumerilii*

Proteinase K, Pronase and Papain, the prime candidates from the previous screen (performed on *Platynereis dumerilii* larvae) were also tested on adult *Platynereis* specimens. Incubation of living worms in enzymatic cocktails even for prolonged periods of time did not yield large enough numbers of cells to be collected and examined. The tough cuticle surrounding adult *Platynereis* adults was not visibly affected after treatment for 2 hours with each enzyme mix. Incubation in presence of chitinase did not appear to have an effect either. Mechanical dissociation using a mortar and pestle alone and in presence of the enzyme mixes were tested (Table 2.2.1. Pronase and Proteinase K seemed to yield more individual cells (Table 2.2.1. Most of the cells obtained however corresponded to coelomocytes (Fig. 2.6F-H). Best results were obtained by smearing suspension of partially dissociated tissue over a 75 μm cell sorter with a plastic pasteur pipette, allowing the obtention of a single cell suspension in the flowthrough (Fig. 2.6A) and non-disaggregated tissue retained by the mesh of the cell sorter. Cells obtained included large round cells (Fig. 2.6B-F). Cell contained pigmented granules (Fig. 2.6I &F). To obtain more cell types, adults were dissected using a scalpels and minced with scissors. Explants were incubated in sterile seawater in a beaker with a magnetic stirrer. In such a manner, muscle and neuron-like cells could be obtained (See Chapter 2).

2.2.2 Dechorionation of embryos (64 Cells)

Attempts to dissociate early stages were attempted with above mentioned enzyme mixes. Cells could be obtained only as early as 6hpf with conventional enzyme mixes (Fig. 2.2.2). The fertilization membrane would appear swollen only as of 4-5hpf or after 2-3hours incubation in enzymatic mix. Therefore, efforts were concentrated on the removal of the fertilization membrane.

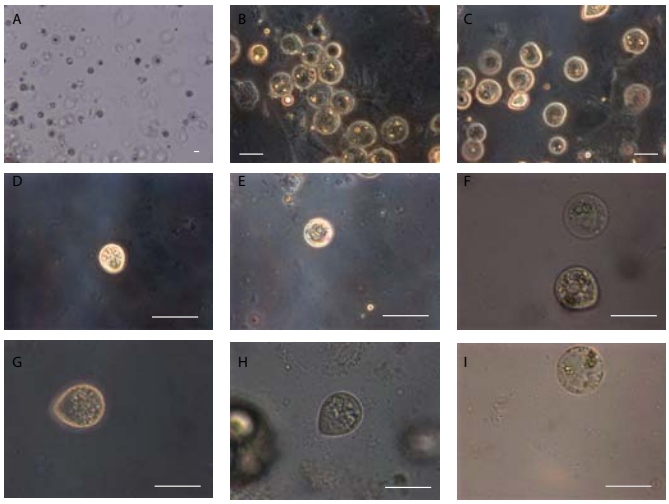


Figure 2.6: Result of dissociation of adult *Platynereis dumerilii*. (A)Passage through a 75 μm cell sorter yielded a heterogenous single cell suspension. (B-C) At higher magnification cells of two primary morphologies could be observed spherical cells and elongated pyramidal cells (D-E)In phase contrast internal granular structures could be visualized. (F & I) In Nomarski optics a large vesicular structure could be observed surrounded by small granular structures. (G-H) Coelomocytes were not observed as lysing of suffering from any form of osmotic shock after enzymatic and mechanical treatment, whereas the large round cells were observed to die(I).

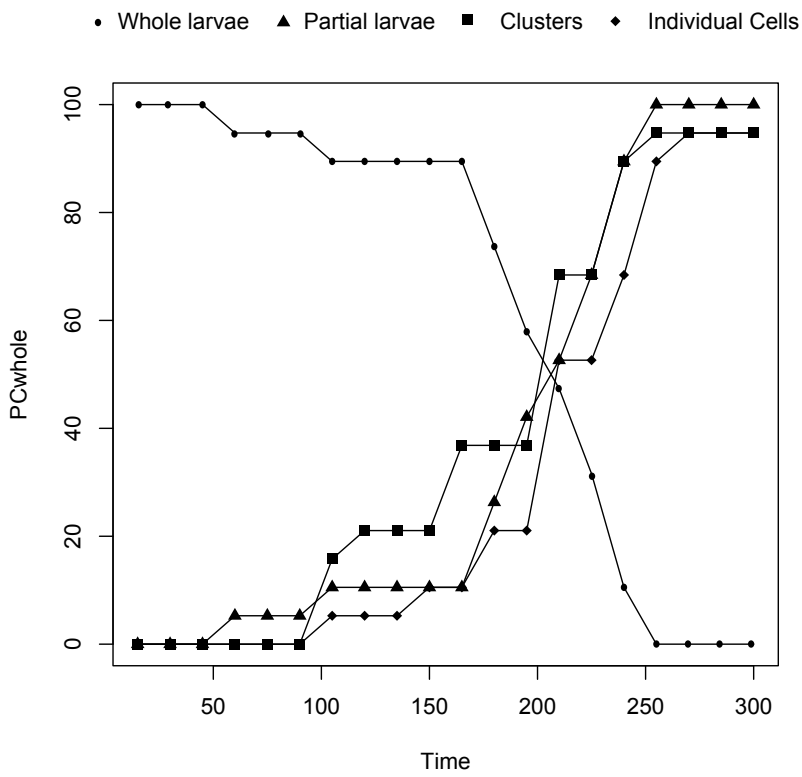


Figure 2.7: Dissociation of Platynereis early blastomeres using Pronase as a function of time. Number of observed whole larvae, partial larvae, cluster of cells and individual cells over 300 minutes.

Table 2.2: Dissociation of adult *Platynereis dumerilii* using mechanical dissociation alone and in presence of enzymatic dissociation solutions

Enzyme	Individual cell disaggregation	Yield or survival
Proteinase K	+++	+++
Pronase	+++	+++
Papain	++	++
Elastase	++	++
Mechanical dissociation alone	++	++

Dechorionation of young larvae by membrane swelling

In the late 19th century "[EB] Wilson stated that his frequent attempts to study the development of isolated blastomeres of *Nereis* were rendered fruitless by "practical difficulties" [147]. Despite this discouraging statement, early 20th century zoologists developed ingenious strategies for the study of polychaete blastomeres. Driesch and Morgan obtained "egg fragments and blastomeres by shaking eggs in test tubes" [148]. However, as noted by Donald Costello, "such a [crude] method is useless for *Nereis*, [because] the membranes cannot be dissected off with forceps. The [fertilization] membrane is so tough and the protoplasm so delicate that it is impossible to insert a needle through the former without cytolyzing the egg". In order to overcome this difficulty the fertilization membrane was elevated and jelly removed by various chemical treatments. Moser treated *Arbacia* eggs with saponin and observed membrane elevation [149, 149]. Lillie described the destruction of the *Chaetopterus* egg vitelline membrane in KCl and $CaCl_2$ solutions [150]. The development of the resulting *Chaetopterus* eggs was "very abnormal under the conditions of these experiments, but no special efforts were made to cultivate such embryos further" [151]. This phenomenon was found to be similar to that observed 50 years later by Catherine Henley when she investigated membrane elevation in *Chaetopterus* eggs using alklaline NaCl. Similarly, Novikoff removed the vitelline membrane from fertilized eggs of *Sabellaria* by treatment with an isotonic solution of NaCl, brought to pH 9.6 by the addition of Na_2HCO_3 [152]. He later demonstrated that the same solution could be used on unfertilized *Nereis* eggs [153]. This treatment had different effects on different species. Treatment of *Chaetopterus* with alkaline NaCl resulted in "denuded" embryos in 5 minutes, whereas Costello observed that NaCl treatment of *Hydroides* eggs results in "the actual dissolution of the *Hydroides* egg membrane in the alkali" [154]. Costello successfully removed the egg membrane by one of two chemical methods either isotonic calcium chloride (0.34M) at pH 6.9-7.0 or using alkaline NaCl solution (Fig. 2.2.2) [154].

Lovelace observed that calcium "chloride produces precocious sperm entrance (as determined by observations on living eggs) and early cleavage when applied to *Nereis* eggs 5 minutes after insemination"[155]. Costello (1949) reported that a treatment with alkaline NaCl (pH 10.3-10.5)n cause the swelling of jelly under this membrane triggers the lifting of the vitelline membrane from the surface of the unfertilized egg [148]. This process also induces a weakening of the egg cortex and massive jelly release, which sometimes cause a deadly damage to the plasma membrane.

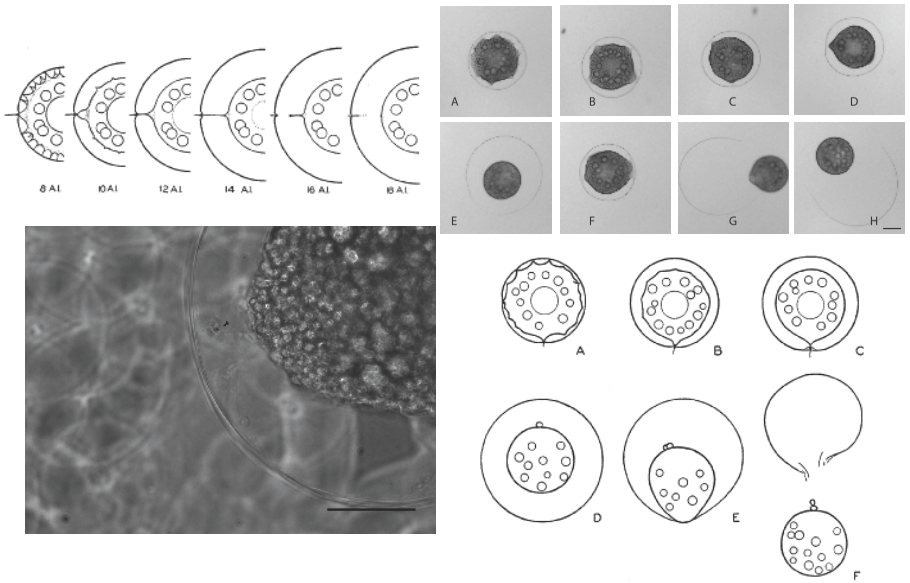


Figure 2.8: *Platynereis* embryo fertilization membrane removal using alkaline NaCl treatment. Top left panel: fertilization membrane swelling as described by Costello. Top right panel: personal observation of membrane detachment of *Platynereis* embryo (Images taken with 2-3 minute intervals) Bottom left panel: Personal observation of membrane detachment at high magnification (40x) - filaments are observed to remain in contact with membrane and slowly detach. Membrane detachment as schematisized by Costello [148]

Costello described the treatment of *Nereis limbata* eggs with alkali NaCl in a 55mm in diameter vessel filled utilizing 25cc of alkali NaCl. Indeed this detail appears to be important when it was attempted to reproduce these experiments. Indeed, treatment with alkali NaCl in 5ml glass dishes was unsuccessful. The membrane swelling and the first embryonic cleavage were observed. However, the release of embryos from the fertilization membrane and jelly was never observed. Eggs obtained from a single fertilization separated into three approximately equal groups and subsequently treated with

1. 25ml alkali NaCl in 2.5cm in diameter 100ml graduated measuring cylinder
2. 25ml alkali NaCl in 4cm in diameter 100ml graduated measuring cylinder
3. 25ml $CaCl_2$ pH 7-8 in a 2.5cm in diameter 100ml graduated measuring cylinder (see below) showed different results

Embryo release was observed in the first and third group however in the second extensive embryonic death occurred. Novikoff claimed that "development ensues in about 50% of the denuded eggs" of *Nereis limbata* [156]. This statement was contested by Costello. The yield of embryos obtained with *Platynereis dumerilii* in my hands was considerably lower than that of either Novikoff or Costello (Table 2.2.2 and 2.2.2). Saponin treatment resulted in brief swelling of larvae followed by rapid larval death.

Table 2.3: Survivors after alkali NaCl treatment

Trial Nb.	Number of survivors	Total number of larvae	Percentage survivors
1	5	312	1.6
2	17	219	7.8
3	3	113	2.7
4	2	347	0.57
5	8	212	3.77
6	4	97	4.12
7	9	131	6.87

Table 2.4: Survivors after $CaCl_2$ treatment

Trial Nb.	Number of survivors	Total number of larvae	Percentage survivors
1	6	50	12
2	0	108	0
3	2	213	0.94

After less drastic treatments, the asymmetrical exaggerated membranes retained their configuration through at least the first cleavage and denuding did not occur.

Dechorionation by dissection

At first, mechanical removal of the fertilization membrane was attempted, however as with EB Wilson, a century ago, attempts were unsuccessful. Therefore, a differ-

ent method was attempted, based on a method developed for *Ciona intestinalis* [157]. Chorions were allowed to swell (Fig. 2.2.2) by brief incubation in double strength seawater and mouth aspirated into a pasteur pipette with tip fire-pulled to a diameter of 200 μm (diameter of *Platynereis* egg). Upon expulsion from tip of pipette a broken tip of another pasteur pipette or a fire polished tungsten needle were used to puncture the swollen chorion. Pipetting of embryo with punctured chorion up and down allowed complete removal of chorion. The procedure is very labor-intensive and in my hands was not efficient (Table 2.5).

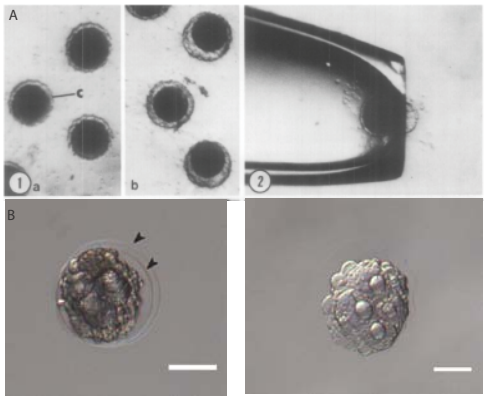


Figure 2.9: Adaptation of *Ciona* dechorionation protocol(A) Procedure as described by Muneoka (Fig.1 and 2 of [157]: "Unfertilized eggs of *Ciona intestinalis* in (a) normal seawater and (b) double-strength artificial seawater. Note the increased space between egg and chorion resulting from the shrinkage of the egg. C, chorion.")(B) *Platynereis* 7hpf larvae with swollen chorion. Black arrows indicate two layers of swollen chorion.

Dechorionation using membrane softening chemicals

As traditional non-damaging methods tested, proved unsuccessful, more aggressive methods were attempted. Several standard methods to soften the fertilization membrane for subsequent staining have routinely been employed in marine invertebrates. Many coats produce sulfhydryl linkages between coat proteins during the hardening process, hence the usefulness of thioglycolate, 3-ATA and para-amino-benzoic acid are often used (Table 2.2.2).

Trial nb.	Successfully dechorionated	Total attempts	Stage
1	12	83	6-8hpf
2	19	179	8-9hpf
3	6	72	4-6 hpf
4	8	89	5-6hpf
5	21	231	9-12hpf
6	17	103	6-7hpf
7	10	84	5-8hpf
8	11	72	6-9hpf
9	9	81	5-10hpf
10	13	113	6-10hpf
11	7	131	4-10hpf
12	5	75	5-8hpf
13	3	23	6-9hpf

Table 2.5: Success rate at dechorionation of *Platynereis* larvae by dissection and osmotic shock[157].

Table 2.6: Chorion softening methods routinely used in marine organisms

Substance used	Phylum	Species	Reference
0.5 M Sucrose & 0.125 M trisodium citrate	Polychaetes	*Sabellaria*	
0,1% protease	Larvacean	*Oikopleura dioica*	
1% protease	Ascidians	*Corella inflate*	
0.1% protease 1% Na-TG pH 10	Ascidians	*Stolidobranch* eggs	
CMF	Bivalves	*Crassostrea, Chlamys*, & *Acila*	
1 mM 3-ATA 1hr	Ophiuroids	*Ophiopholis*	
1% Na TG pH 10+protease	Ascidians		
0.1% pronase	Asteroids	*Pisaster*	
1mM 3-ATA in NSW	Echinoderms		
1mM 3-ATA in NSW	Shrimp	*Sicyonia*	
0.87M Sucrose	Echiurans	*Urechis caupo*	

These methods are applied in order to soften fertilization membranes and other coats enveloping young embryos for subsequent stainings, rather than for dissociation purposes. All embryos incubated in 3-ATA briefly after fertilization remained unfertilized. Saponin treatment resulted in embryonic death. Sucrose treatment resulted in observed osmotic shock and stalled development (Table 2.2.2).

Table 2.7: Results of chemical treatments attempted to soften fertilization membrane

Method	Dechorionation	Survival
Manual chorion removal	+	-
Osmotic shock	+	+
Saponin	-	-
Alkali NaCl	+	+/-
$CaCl_2$	+	+/-
3-ATA	-	-
Sucrose (0.87M)	-	+
Sucrose in SFCMFSW	-	+
Sucrose and sodium tricitrate	-	+
Sodium thioglycolate 0.5%	-	+

Dechorionation using membrane softening chemicals and enzymatic treatment

As Papain, Pronase and Proteinase K had performed best in assays to dissociate older larvae, these enzymes were utilized in combination with the membrane softening chemicals described above (section 2.2.2) in SFNSW and SFCMFSW.

Table 2.8: Results of chemical treatments attempted to soften fertilization membrane

Additive	Papain	Pronase	Proteinase K
Osmotic shock	-	-	-
Alkali NaCl	-	-	-
$CaCl_2$	-	-	-
3-ATA	-	-	-
Sucrose (0.87M)	-	-	-
Sucrose and sodium tricitrate	-	-	-
Sodium thioglycolate 1%	N/A	+++	+

Curiously, incubation of embryos beginning cleavage or at 2-cell stage in a solution of 0.1%- 5%of sodium thioglycolate in SFNSW did not appear to visibly affect chorion formation. Addition of Pronase 0.05% to 0.1% to a solution of 1% sodium thioglycolate in SFNSW did not affect chorion removal. Incubation of embryos in 1% sodium thioglycolate in SFCMFSW with trace amounts of pronase (0.05%) shaking in a 6 well plate on an eppendorf Thermomixer successfully affected chorion permeabilization. Incubation with or without shaking for periods above 30 minutes resulted in cell lysis. Transfer of embryos with visibly swollen fertilization membranes into fresh calcium-magnesium free seawater after 10 minutes incubation in Sodium thioglycolate

Table 2.9: Comparison of blastomere image quantification to published data

Blastomere	Dorresteijn 1990			Present evaluation		
	% total egg volume	SD	n	% total egg volume	SD	n
2-cell stage						
AB	26.74	0.95	7	28.09	10.67	16
CD	73.26	0.95	7	71.831	16.65	16
3-cell stage						
A/B	N/A	N/A	N/A	28.32	6.89	94
CD	N/A	N/A	N/A	41.922	10.20	47
4-cell stage						
A/B	13.445	1.52	3	12.34	9.59	22
C	22.07	2.08	3	31.91	7.56	11
D	51.04	1.84	3	46.73	6.40	11

and Pronase mix alone gentle dechorionation using a mouth pipette capillary. Pipetting the cleaving embryo up and down allowed removal of chorion and dissociation into individual cells (Fig. 2.10).

Early embryonic cleavage and cell fate determination is a well studied and very delicate process. As shaking and enzymatic treatment could affect the ”cell type”, embryos were assayed based on criteria described for young *Platynereis* larvae [158]. As *Platynereis* is a spiralian cleaving organism, its first embryonic cell divisions are stereotypical. After 90-100 minutes (at room temperature), ooplasmic movements can be observed in fertilized larvae. Within 15 minutes, the first cell division occurs, generating two daughter cells: a smaller AB cell and larger CD cell (Fig. 2.11A). Fifteen minutes (at room temperature under a binocular), a second cell division occurs. The AB cell divides into two daughter cells of almost equal size: the A and the B cell (Fig. 2.11B). Morphologically these cells are practically indistinguishable (Fig. 2.11). This cell division is followed by the division of the CD cell into a slightly smaller cell C and a larger cell D. The D cell characteristically contains clear cytoplasm which is hypothesized to contain cell fate determining molecules [158].

Dorresteijn quantified the relative sizes of each of the cells and the amount of clear cytoplasm using a labor-intensive protocol involving fixation, embedding and dehydration of larvae. In order to test similar properties could be approximated using light microscopy, images of *Platynereis* non-dissociated larva were taken and relative sizes were compared to the data described by Dorresteijn [158, 159]. Indeed, data obtained using light microscopy was similar to that obtained by Dorresteijn (Fig. 2.2.2).

Platynereis larvae incubated Pronase and Sodium thioglycolate solutions on shakers frequently appeared to cleave in an aberrant fashion (Fig. 2.12). To evaluate whether *Platynereis* cell division was affected by mechanical agitation in presence of enzymes, images of obtained cells were taken and by the method described above compared to non-treated siblings (Table 2.2.2) . Furthermore, several times fresh solutions composed of calcium magnesium free seawater appeared to affect development. As the chorion was not observed to swell (a sign of osmotic shock), the culprit was hypothesized to be the pH. To evaluate whether pH affected *Platynereis* cell division, the same

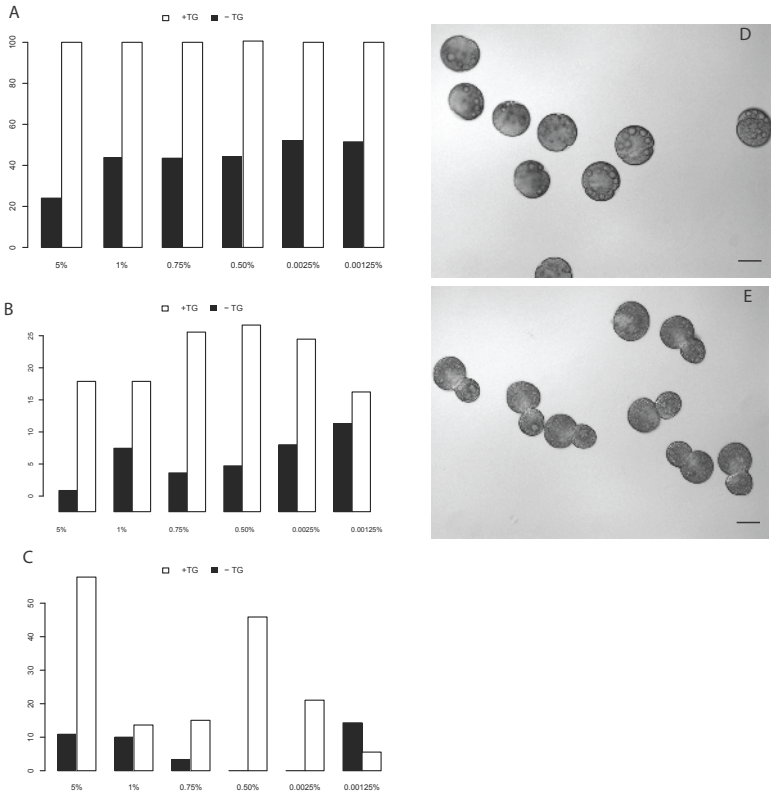


Figure 2.10: Different concentrations of Pronase in presence and absence of Sodium Thioglycolate (TG) after incubation for 15minutes. (A-C):(A)Percentage larvae with swollen or absent chorion. (B) Number of individual detached cells observed after 15 minutes incubation (C)Percentage larvae with lysed cells. (D) Larvae incubated in 0,25% Pronase after 7 minutes in Pronase alone. (E) Larvae incubated in 0,25% Pronase and 1% Sodium thioglycolate after 15 minute.

protocol was applied and larvae were allowed to develop in NaCl adjusted to different pH values (2, 4, 6, non-adjusted 6.5, 9, 10).

Figure 2.11 *(following page)*: (A)*Platynereis* 1-cell stage embryo. (B) *Platynereis* 2-cell stage embryo (C) *Platynereis* 2-cell stage embryo showing nuclear division of AB cell nucleus (white arrow). Cell membranes are stained with phalloidin (green) and DNA with DAPI (blue)(D) *Platynereis* 2-cell stage embryo with 2 distinct nuclei in AB cell (red arrows) (E-F) 4-cell stage embryos undergoing nuclear division (white arrows indicate nuclear division)

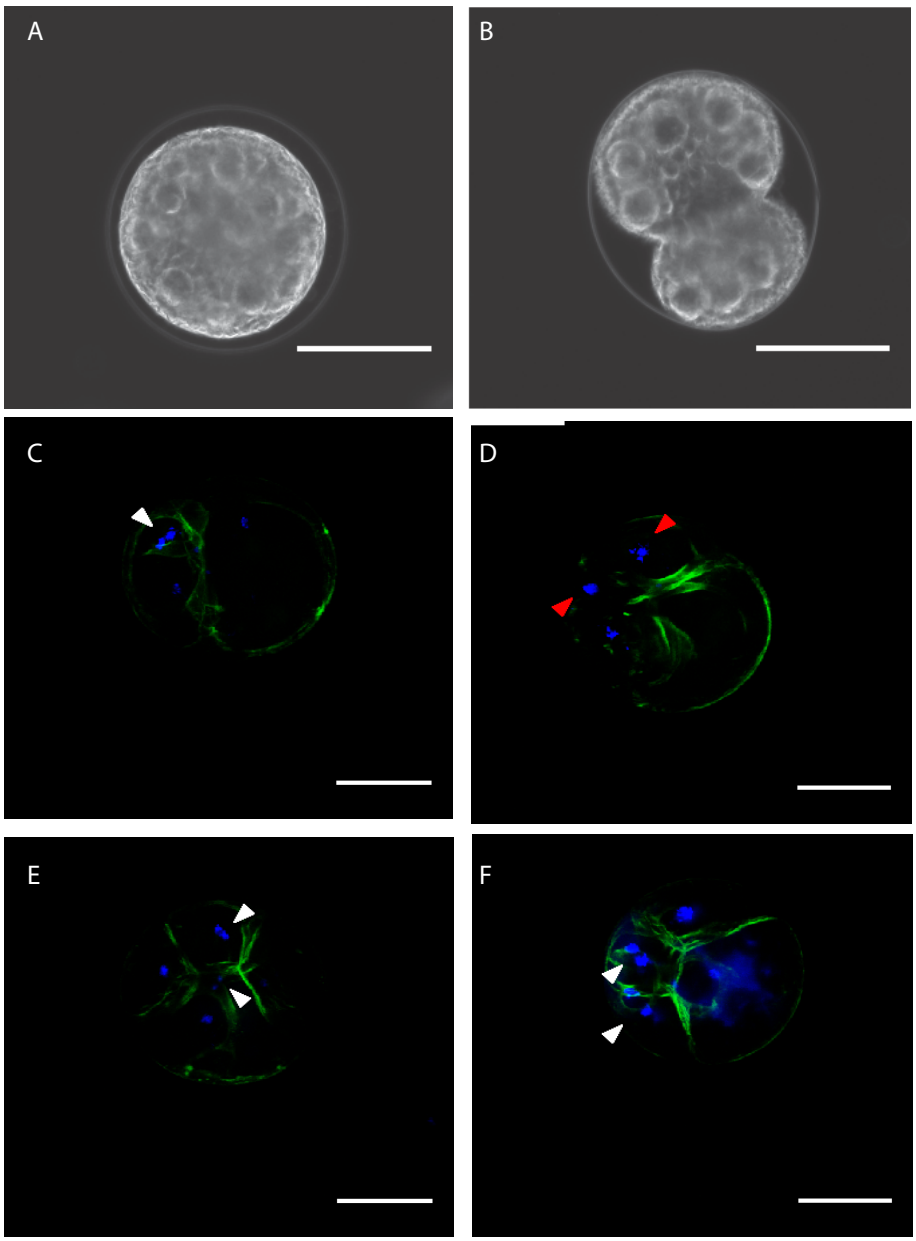

A
B
C
D
E
F

Figure 2.12 *(following page)*: Top row: 1-cell stage larvae, second row: 2-cell stage larvae, third row: 4-cell stage larvae. Left column: non-dissociated larvae with chorion, second column from the left: dechorionated larvae treated with Pronase 0,25% and 1%Sodium thioglycolate, three columns to the right: diversity of misdevelopers observed after dechorionation. Transparent cytoplasm putatively rich in fate determining factors (black arrows).Bottom row: cytoplasmic prolongations (white arrows) produced by Pronase and Sodium thioglycolate treated blastomeres.

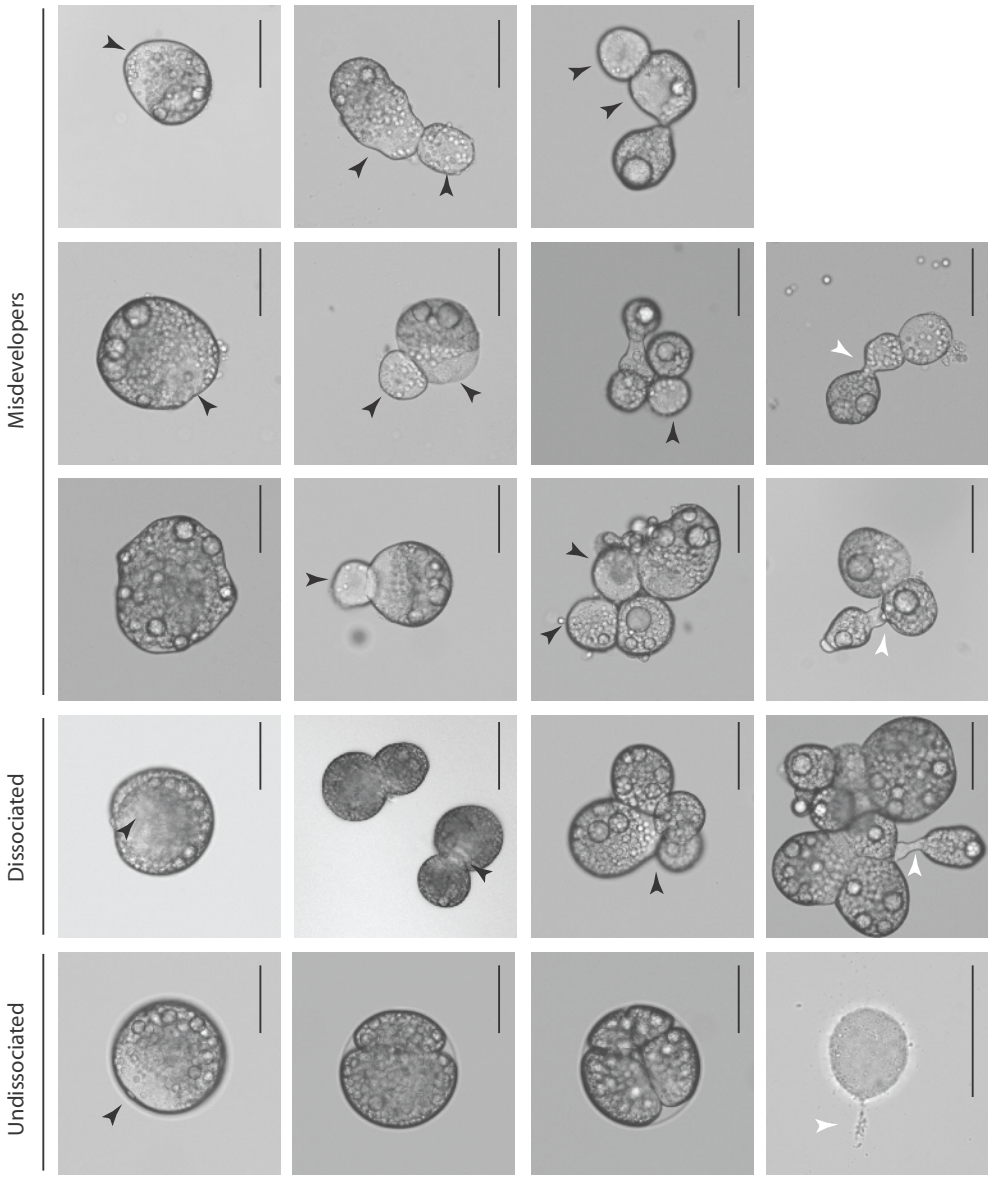

Misdevelopers
Dissociated
Undissociated

Table 2.10: Comparison of blastomere image quantification in presence of mechanical agitation to published data

Blastomere	Dorresteijn 1990		Present study after agitation	
	% clear cytoplasm	% yolk	%clear cytoplasm	% yolk
AB	22.32	55.63	54.2	55.37
CD	41	69.9	47	63.21
3-cell stage				
A/B	23.9	58.47	79.3	18.67
C	30.78	72.89	14	23.98
D	46.85	66.50	14.39	11.67

To confirm that intracellular component segregation was not affected, phosphotungstic acid staining (stains proteins [160, 161, 162]) was performed (Fig. 2.2.2). WMISH using polyT probes was unsuccessful in demonstrating RNA segregation, as signal was observed in the entire egg.

Furthermore, in order to ascertain that properties of embryonic *Platynereis* cells were maintained, dissociated blastomeres were observed using 4D microscopy. Cell lineage was followed (Chapter 3).

2.3 Cell separation

Multicellular organisms are composed of a variety of cells. Once dissociated, sorting the resulting cells based on their properties is an important step to characterization. Numerous methods have been used for this purpose. A manual method with the advantage of visualizing each cell selected, has the disadvantage of being labor-intensive and time-consuming. Filter-based methods, are more rapid, but do not allow to make sure of the quality of the sorting, making possible much contamination. Density gradient centrifugation is based on more properties of the cell than size, such as density and buoyancy. However, this method is technically challenging and can be deleterious to cells. Flow cytometry is a very efficient method, that requires much calibration and requires large amounts of starting material for such calibrations. I have attempted each of these approaches with variable success.

Cell sorting using nylon filters

Attempts were made to sort cells using a set of different nylon filters with different mesh sizes: 10, 15, 20, 25, 75 (cell sorter mesh), 100, 150 and 300 microμm in diameter. Mesh fragments inserted in P1000 micropipettes tips were placed successively one on top of each other and a cell in decreasing order of mesh size. This procedure albeit simple was highly inefficient. Small cells were not retained by even the 10 microμm meshes and cell clumps consisting of aggregates of small cells which collectively composed an aggregate of over 100 microμm were retained in higher mesh size filters. Furthermore, any contamination or debris from the dissociation solution rapidly

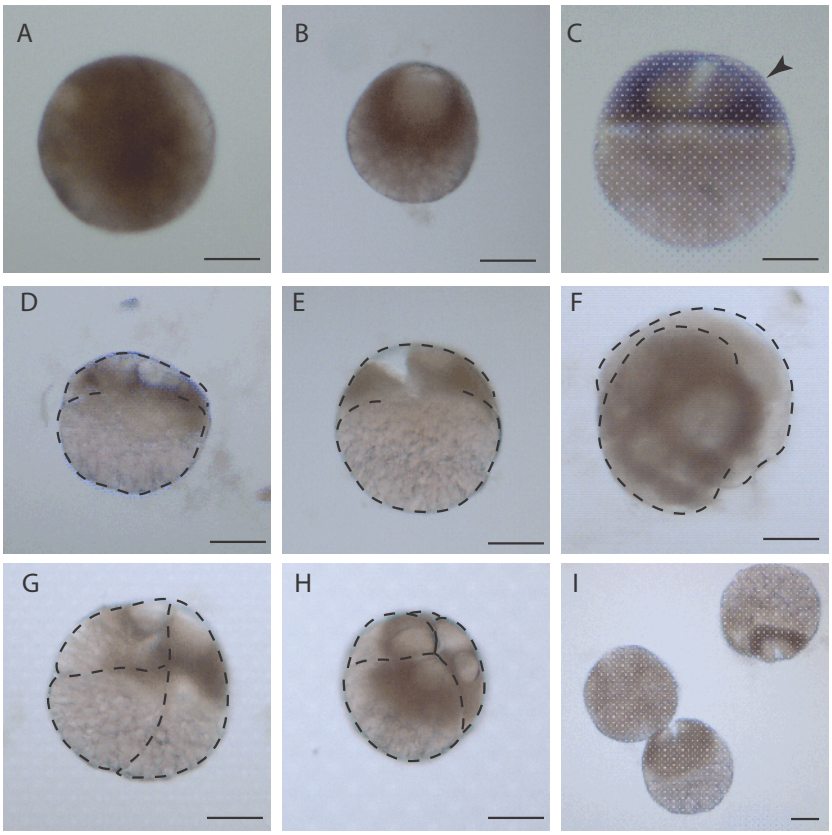


Figure 2.13: Phosphotungstic acid staining of *Platynereis* early embryos as observed 30 minutes after incubation In 1-cell stage embryos protein segregation in the cytoplasm can be observed over time (A-B). Early embryos condense their cytoplasm into three different types as defined by Phosphotungstic acid staining: Dark staining, granular staining and light or no staining (A-I) Within a few minutes embryos are seen to segregate cytoplasm such that the clear non-stained cytoplasm is localized to one side of the egg, surrounded by a band of dark staining. The opposite pole of the egg corresponds to the granular cytoplasm (B). Upon treatment, the first staining appear violet (C) and progressively become brown over time. As cell division is followed the darker stained portion evidently becomes localized to one cell and the granular-stained portion to another (D-F). The clear and faintly stained pattern observed at one pole of the egg remains at this end and becomes more clearly defined as 4 distinct structures, leading to believe that they may correspond to the lipid droplets(G-H) A diversity of staining intensities was observed (I) Scale bar: 50 μm

clogged the entire setup. Although, cell populations of a certain size could be enriched by this procedure, pure single size range cell populations could never be obtained.

Cell separation by density gradient centrifugation

A variety of cell types exist within single organisms. These cells differ in their size and morphology. A difference is also observed in their relative densities and buoyancy. Furthermore, source organisms are not bread in sceptic conditions, and the presence of bacteria, fungus and protozoan contaminants is likely. In order to separate cells from each other and eventual contaminants, attempts were made at density gradient centrifugation, using Ficoll and Percoll solutions. Density Gradient Separation is method used for isolating and purifying cells, viruses and subcellular particles. By centrifuging the cells in a solution containing particles that form a gradient according to their sedimentation rate, cells can be separated according to their density (isopycnic centrifugation) or size (rate zonal centrifugation). Ficoll is a neutral, high mass hydrophilic polysaccharide which dissolves in aqueous solutions. Percoll is made of colloidal silica particles (15-30 nm diameter) coated with non-dialyzable polyvinylpyrrolidone (PVP). Due to its low viscosity and low osmolarity (25 mOsm/kg) it is considered relatively non-toxic to cells.

Sedimentation of dissociated in cells resuspended in NSW or Calcium-Magnesium Free Seawater (CMFSW) was attempted with both reagents in gradients ranging from 40% (Ficoll or Percoll) to 0%. Cell survival was achieved only upon utilization of a concentrated stock solution of CMFSW for the preparation of each layer of the gradient.

Density gradient centrifugation using Percoll reagent was used by Heacox [163] to isolate *Nereis virens* oocytes from other cell types in for the subsequent culture of the oocytes. A similar approach was adopted. By using a gradient in steps of 2% ranging from 16% Ficoll to 2% and adding 500 μl of eggs derived from a recent fertilization and letting eggs sink (without centrifugation) through the gradient, two distinct layers of eggs were rapidly formed. The top layer constituted the less dense and lower more dense layer. Eggs from each layer were collected and washed with NSW to remove Ficoll and both groups of eggs maintained in NSW for 24 hours. All individuals obtained from the lower layer were observed to be dead the following day. Swimming larvae were observed in wells in which eggs from the upper layer were observed.

This is in line with the results obtained by Devauchelle, Dorange and Faure[164]. The authors used Percoll gradients to separate viable embryos of the scallop, *Pecten maximus* from misdeveloppers.

Furthermore, using the protocol used by Odintsova [165] (Fig. 2.3) for the isolation of mussel muscle, neuron and ciliated cells, a portion of the cells obtained from dissociation was isolated and used for culture. Although, this method did not yield the same results upon culture, ciliated prototroch cells and a number of large cells, which include unidentified cells, macromeres, some small cells and cells with specific morphology could be isolated. This method also had the advantage of efficiently separating cells from bacteria which remained at the top of the gradient (Fraction 1 or 10%), whilst *Platynereis* derived cells sank through (Fig. 2.3. Fraction 2 contained the larger cells and cell aggregates (on average over 70 microμm in diameter). Fraction 3 contained

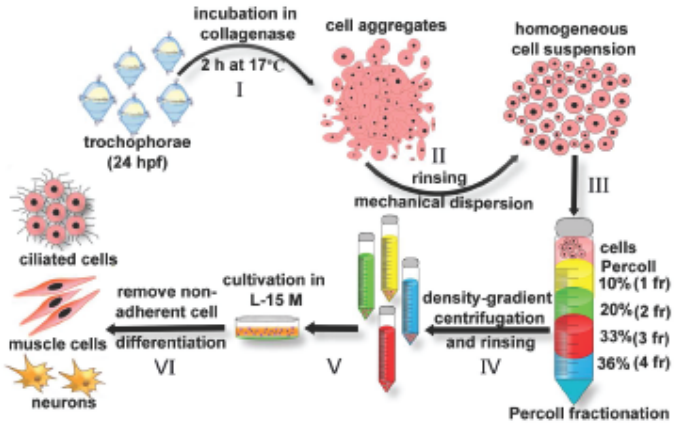


Figure 2.14: Figure reproduced from Odintsova *et al*., 2010. Original caption: Schematic representation of the experimental procedure of establishing the trochophore-derived primary cell culture of *M. trossulus* (24 hpf). Trochophore larvae were collected and dissociated by an enzymatic treatment (I). Dissociated larval cells were rinsed (II) and fractionated on Percoll density gradient (10% - 36% Percoll: 1 fraction (fr), 2 fr, 3 fr, 4 fr) (III). After washing out the Percoll medium (IV), the cells were plated on glass coverslips and cultured in the L-15M medium [133] overnight at 17C (V). Then, nutrient medium was changed to fresh one.The non-adherent cells were removed in the time of medium change (VI)."

small-sized aggregates of small cells. Fraction 4 (36% Percoll reagent) did not contain any aggregates, no large aggregates composed of a multitude of cells, but rather of a aggregates of few large cells. Non-circular morphology was observed among the cells obtained from this fraction. Fraction 4 contained only small cells (Fig. 2.3.

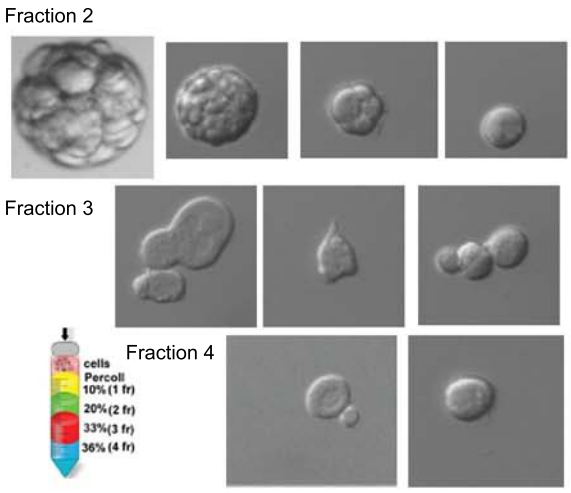


Figure 2.15: Cells separated using the protocol utilized by Odintsova [165] for the isolation of mussel muscle, neuron and ciliated cells as described in Fig. 2.3. Fraction 2 (20% Percoll reagent) contained large cells and large cell aggregates (over 70 μm in diameter). Fraction 3 (33% Percoll reagent) contained small-sized aggregates of small cells, whereas Fraction 4 (36% Percoll reagent) contained small cells only. No aggregates were observed. These cells in some cases displayed cilia-like structures and eolongated

Cell separation by flow cytometry

Attempts were also made to sort cells using Flow cytometry, without fluorescent staining or reporter genes, based purely on cell size. This approach has been fruitful in planarian cell sorting [166]. Dissociated *Platynereis dumerilii* blastomere (4-cell stage) were used for this approach, as the difference in cell size was deemed considerable and should allow better sorting possibilities than later stages when most cells are smaller and it is harder to distinguish between them based on size only. In order to avoid osmotic shock the setup was perfused with 20L 1xSFCMFSW. Furthermore, using the FloMo flow cytometer (Beckman Coulter), non-dissociated embryo sorting was attempted. Experiments were carried out by Dr. Andrew Riddell and Dr. Alexis Perez Gonzalez from the EMBL Heidelberg Flow Cytometry facility. Details of machine

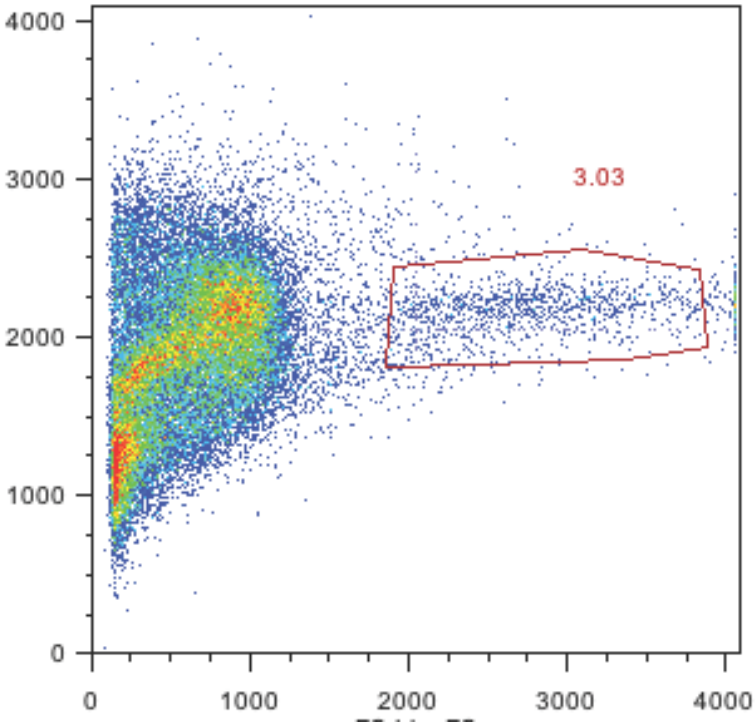


Figure 2.16: Scatterplot of particle distribution. Red box indicates fraction containing *Platynereis* blastomere cells. Particles to the left indicate background from lyzed cell debris. Y-axis: Side scater log X-axis: Forward Scatter lin

function and setup can be found in Appendix I. The text and figures enclosed were produced by Dr. Alexis Perez Gonzalez (Fig. 2.3).

This approach is very promising. However, the fine tuning requires considerable amounts of material (10^6 cells). Using *Platynereis* cells, this amount of starting material is difficult to obtain. All the attempts at cell sorting were —performed with approximately 4000 cells. Considerable background was observed due to lipid droplets liberated from lyzing cells. Indeed, a considerable portion of the material sorted at initial steps corresponded to lipid droplets only. Attempts to stain live cells using Hoechst live DNA dye, was not helpful, as the dye also bound DNA in solution from lyzed cells.

Cell sorting using mouth pipetting and capillary

The easiest method attempted made use of a system routinely utilized in mouse transgenesis, consisting of a pulled capillary placed in a capillary holder inserted in a piece of rubber tubing. The tubing is interrupted in the middle by a 0,2 microμm syringe filter.

At the other end of the tubing is enclose a mouthpiece. Alternatively, a micropipette-tip can be used. The mechanism is detailed in [167]. This method is the most labor-intensive method tested. It is also the least sterile and time-consuming. Unfortunately, this method was the most efficient among those tested.

2.4 Prospects

The goal of the experiments described above was three-fold:

1. to provide a reliable and simple method for dissociation of *Platynereis dumerilii* larvae, embryos as well as adults into individual cells
2. to establish qualitative and quantitative methods to evaluate the degree success of the dissociation procedure
3. to establish methods to sort cells resulting from the dissociation procedure

A preliminary qualitative screen to determine the best conditions for the dissociation of *Platynereis* larvae (staged at 24hpf and 48hpf) was performed using 11 enzymatic solutions containing either : Trypsin, Collagenase, Collagenase/Dispase, Proteinase K, Pronase, Pronase & Collagenase/Dispase, Papain, Elastase or Lysozyme at concentrations ranging from 5%, 1% and 0.075-0.025% and using several methods for mechanical dissociation. The efficiency each solution was first of all evaluated qualitatively and thereafter quantitatively. The qualitative screen eliminated candidate dissociation procedures utilizig Trypsin, Collagnase, Elastase, Lysozyme and purely mechanical trituration as dissociation was judged as inefficient. Trypsin was deemed deleterious to *Platynereis* cells. The deleterious effect of Trypsin has been observed in other organisms. Trypsin EDTA has often been used to dissociate cells from the digestive gland of *M.galloprincialis*, *Crassostrea gigas*, *Ostrea edulis* and *Mytilus edulis*. At low concentrations Trypsin-EDTA caused digestive cells to burst leaving behind lipid droplets and many residual bodies [130]. In order to carry out a quantitative evaluation of enzymatic dissociation efficiency, quantitative assays had to be established on *Platynereis* cells. Four functional tests were carried out. The capacity of FDA, MTT, NR and TP assays to measure the viability and functional activities of non-specific esterases, mitochondrial dehydrogenases and lysosomal incorporation and capacity to exclude TP were evaluated, demonstrating that:

1. *Platynereis* blastomeres and larval cells from 24 and 48hpf larvae were capable if de-esterifying FDA, transforming MTT, incorporating NR and excluding TP
2. that these assays could be carried out successfully with low amounts of cells
3. these tests could be rapid and sensitive in cell culture conditions

More specifically, MTT and FDA respectively measured the viability during a period of two hours after dissociation and immediately after the dissociation procedure. NR incorporation and TP exclusion showed similar trends to those observed by simply counting cells using a hemocytometer. MTT, FDA, NR and TP assays did not

show an identical trend. This observation could indicate that either different aspects of *Platynereis* metabolism are affected differently by proteolytic treatment or that the assays were irreproducible. Repeating these treatments at higher sample size or sample number could help determine whether which is the case, however this was deemed not feasible due to the limitations in terms of starting material available. The overall view indicates that Papain appeared to perform most efficiently in terms of some parameters (MTT) and more poorly in other parameters, whereas Proteinase K performed most poorly in terms of all parameters tested. Pronase on the other hand performed satisfactorily "in-between". Furthermore, Pronase could be easily resuspended as compared to Papain. For these reasons, Pronase was selected for all subsequent dissociation reactions performed on trochophore and nectochaete larvae.

Subsequently, efforts were applied at denudation of early cleaving embryos. Although Pronase proved satisfactory at the dissociation of larvae, Pronase alone required several hours to remove the chorion (Fig. 2.2.2). Therefore several "denudation" methods described in literature were tested. Alkali NaCl and $CaCl_2$ treatment described by Costello and Novikoff for *Nereis limbata* proved far less efficient at *Platynereis* denudation providing a disappointingly low yield (Table 2.2.2 and Table 2.2.2). Manual denudation using osmotic shock [157] as means to swell the membrane proved laborious and provided a disappointing yield (Table 2.5). A small scale screen using a variety of membrane softening (Table 2.2.2) chemicals utilized in membrane softening or dissolution in a variety of marine invertebrates was carried out (Table 2.2.2), including Saponin, 3-ATA, Sucrose 0.87M, Sucrose and sodium tricitrate and Sodium thioglycolate. However, results proved only further disappointing. For this reason, a combination of proteolytic enzymes and of chemicals were tested together. Based on the original qualitative and quantitative dissociation screens performed on larvae Papain, Pronase and Proteinase K were selected for evaluation. Of the seven methods tested (osmotic shock using a concentrated seawater solution, alkali NaCl, $CaCl_2$, sucrose 0.87M, Sucrose and sodium tricitrate and sodium thioglycolate), only Sodium thioglycolate in presence of proteolytic enzymes proved efficient (Fig. 2.10). The optimal concentration of sodium thioglycolate and enzyme was determined by testing different concentrations over a period of 15 minutes.

The importance of the medium in which digestions are performed is often underestimated. Calcium, magnesium and sulfur cations are involved in membrane links. Addition of 12mM of each of the latter up to the level determined in the hemolymph of bivalves has been shown to be delay cell release from acinar structures of *Pecten maximus* [130]. This was particularly important for the dechorionation of young embryos. Several methods described in literature were attempted, however only one method was deemed satisfactory, the combined action of Pronase and Sodium thioglycolate in SFCMFSW.

The effectiveness of the dissociation solution on embryos and larvae at different developmental stages was also investigated. Sodium thioglycolate was judged essentially important for the denudation of early cleaving embryos. Dissociation of older larvae could be achieved in the absence of Sodium thioglycolate, however chorion removal could not be achieved in the absence of Sodium thioglycolate before several hours. This indicates that peptide linkages involving disulphide bonds are important in *Platynereis* chorion membranes in early development, but are less present in older

larvae. Sodium thioglycolate has been extensively used for embryo denudation in other organisms such as *Rana pipiens* [168].

Furthermore, criteria were established to confirm morphologically that cells were not deleteriously affected by the dissociation process (Table 2.2.2). As the early embryonic cleavage, development and lineage of *Platynereis* embryos has been characterized by Dorresteijn [169, 158, 170] and colleagues and Ackermann and colleagues [171], "normal" development could be assayed using morphological criteria. Non-treated embryos were imaged using light microscopy and compared to published data. The amount of clear cytoplasm putatively containing cell fate determining factors was measured using light microscopy. As *Platynereis* is a spiral-cleaving organism, respective cell sizes play an important role in subsequent cell fate determination. Dechorionated embryos were imaged and respective cell sizes and amounts of clear cytoplasm were quantified, demonstrating that excessively large cells contained inappropriate distributions of clear or non-granular cytoplasm. Mechanical agitation accelerated denudation using the Pronase and Sodium thioglycolate solution. However, image quantification of cell size and relative clear cytoplasm demonstrated inappropriate ooplasmic segregation between cells. To confirm that Sodium thioglycolate and Pronase did not affect ooplasmic segregation, a second test was performed utilizing phosphotungstic acid staining (a protein stain - Fig. 2.2.2). This method has not previously been employed in *Platynereis* development and was first evaluated on non-treated embryos. Phosphotungstic acid staining confirmed that (a) protein content of early *Platynereis* cleaving embryos could be stained (b) protein was localized in the egg prior to the first embryonic cell division moving from an equatorial position to an apical location (c) Sodium thioglycolate and Pronase treatment did not affect protein segregation during *Platynereis* early cleavage. Asymmetric protein segregation in *Platynereis* is in line with results from Schneider and colleagues who have demonstrated that blastomere cell fate is dependent on nuclear β catenin distribution during asymmetric cell division in early development. Beginning at the 8-cell-stage β catenin is distributed asymmetrically along the animal-vegetal axis. Moreover, authors demonstrated that ectopic activation of β catenin leads to adoption of sister-cell fate [172].

Several attempts were carried out at performing Wholemount In Situ Hybridization (WMISH) on *Platynereis* early cleaving eggs using Poly T probes, aimed at staining all cellular polyadenylated transcripts. This approach proved unsuccessful, as no specific localization could be observed. This may indicate that transcripts are distributed over the entire egg or that the protocol utilized (NBT/BCIP precipitation reaction) was not adapted to the observation of RNA localization in early *Platynereis* eggs. Further effort was not put into establishing a protocol for early *Platynereis* WMISH as protein staining and clear cytoplasm localization was judged sufficient evidence, of correct development.

Furthermore, although most effort was put into the dissociation of larval and embryonic stages, attempts were made at dissociating adult *Platynereis*. Mechanical trituration was judged most effective as incubation in enzymatic solutions alone did not damage the cuticle surrounding the animals. Mechanical trituration combined with Pronase and Proteinase K action resulted yielded the best results.

Once individual cells from different stages of *Platynereis* development could be obtained confidently, attempts were made at sorting cells using flow cytometry, nylon

filters of varying diameter mesh size, density gradient centrifugation and by manually picking cells of interest using a capillary. Flow cytometry although a very promising technique was unsuccessful in distinguishing between blastomeres based solely on size. Individual non-fertilized oocytes could be counted with varying efficiency. Difficulty stemmed from the low amount of material and the absence of a fluorescent marker which could be used to select cells. Due to the difficulty in obtaining large samples of material fluorescent protein-labeled cell sorting may succumb to the same fate. Indeed, perhaps fixed fluorescently labeled cells (using antibody staining for example) could allow sufficient material for configuration of the equipment and for the obtention of desired cells. Live cells lyze and die in solution, creating extensive background due to lipid droplet liberation. As lipid droplets are less dense, an additional mild centrifugation step may allow the separation of contaminating lipid droplets. Centrifugation at high velocities may result in further cell damage, creating even more background. Density gradient centrifugation allowed the separation of fertilized embryos from non-fertilized embryos based on their buoyancy using Ficoll gradients. Percoll gradients as described by Odintsova when applied to *Platynereis* did not yield as spectacular results as those observed in mussels. Neither neurons nor muscle cells were obtained. Finally, the manual selection of cells of interest was deemed most reliable.

In summary, what may appear at first as a trivial matter, of dissociating young larvae, embryos and adult specimens and subsequently sorting them by morphology, methods that can be confidently considered as established for current "model" organisms since several decades was hindered by paucity of starting material and lack of literature on the topic. Existing literature describing "denudation" methods proved highly unreliable although described as successful in related species of polychaete worms. A reliable protocol for the denudation of chorions from young embryos as well as the dissociation of trochophore larvae and adults were established. Humble attempts were made at cell sorting using several methods, including flow cytometry, density gradient centrifugation, nylon mesh filtering and manual sorting. Finally manual triage of cells with a capillary was deemed most efficient. With these humble methods stably established one can proceed to the characterization the cells obtained.

2.5 Materials and Method

Animal Care Embryos were obtained from an in-house culture of *Platynereis*, maintained by Ms. Diana Bryant as described by [173]. Animals were grown at $18\,^{\circ}\mathrm{C}$ following a light-dark cycle resembling that of the natural moon cycle: approximately one week of artificial moonlight and three weeks of darkness. Mature male and female adults were allowed to spawn and their eggs collected. For young embryo/blastomere dissociation, jelly surrounding the fertilized oocytes was removed after one hour post-fertilization using a 100 μm-diameter mesh. When dissociating trochophore larvae, larvae were allowed to hatch out of the jelly on their own and the remaining non-hatched larvae were aspirated using a plastic pasteur pipette. After jelly removal, larvae and embryos were transferred to SFNSW or SFCMFSW.

Embryo dissociation To perform enzymatic digestion tests, enzymes were prepared as 5% stock solutions. Trypsin and Papain were resuspended in 0.25M DTT and subsequently diluted using stock solutions of SFCMFSW. All other enzymes were resusupended directly in seawater. Additives were premixed at the concentrations described in the text body.

To perform the Muneoka protocol, larvae were incubated in concentrated solutions of SFCMFSW. Tungsten wire obtained from Electron Microscopy Sciences (PA, USA) was electrolytically sharpened. Capillaries were pulled over a bunsen burner.

To repeat the experiment described by Costello, *Platynereis dumerilii* eggs were fertilized by a single male. 5 minutes after fertilization, eggs were transfered from the the jam cup to a glass container and supplemented with alkaline NaCl pH 10.5 (0.53M NaCl, supplemented with 1g Na_2HCO_3) and swirled gently. Eggs were allowed to settle and a fresh solution was added. Eggs were incubated in alkaline NaCl solution for various periods of time. pH was neutralized either by addition of fresh filtered natural seawater, filtered natural seawater pH adjusted to pH 3 (11 drops 32% HCl) or by addition of "acid NaCl" (0.53M NaCl pH adjusted with HCl to pH 2.4 as described by Costello). Eggs were repeatedly washed for several hours with fresh SFNSW.

Trochophore larva dissociation To perform enzymatic treatment, larvae were counted manually, aliquoting 100 larvae per eppendorf, centrifuged at 1000 rpm and supernatant was removed and enzymatic mixes were added to 200 microμl. Serial dilutions of 5% stock solutions were used for the different concentrations of enzyme. Treatments were carried out for 1 or 3 hours. Once the incubation was finished, the resulting solution was filtered through a 75 microμm nylon filter, using an extra 2 mL seawater to wash. The resulting 2.2 mL solution was centrifuged again at 1000 rpm. The supernatant was removed and cells were resuspended in 200 μl fresh SFNSW free of enzyme.

Adult dissociation Immature adults were obtained from the in-house culture. Solutions were prepared as for trochophore larva treatment. Worms were placed in an eppendorf. All excess liquid was removed and a pestle was used to grind the animals. Enzyme solutions were added to ground animals and incubated on a shaker on an Eppendorf Thermomixer at 500-750rpm. Alternatively animals were minced using a scalpel or dissection scissors (generously provided by A.Fischer) and incubated with a magnetic stirrer in a 600 mL beaker. Resulting solution was smeared on a 75 μm cell sorter and the flowthrough examined.

MTT, FDA, NR, TP and total cell number assay 10 μl of MTT reagent (Sigma), or 5 μl of FDA 5mg/ml stock (Sigma), or 0,1% vol. NR, or 0,5% TP were added to 200 μl in a 96 well. MTT absorption was measured with a luminometer at 450nm and 620nm. The 620nm value was subtracted from the 450nm value. FDA fluorescence was measured using a luminometer at 520nm. For NR, TP and total cell counts, cells were resuspended in 60μl instead of 200μl and counted under a hemocytometer.

Chapter 3

Cell characterization: From morphology to single cell transcriptomics

3.1 Introduction

> I have been trying to think of the earth as a kind of organism, but it is no go. I cannot think of it this way. It is too big, too complex, with too many working parts lacking visible connections. The other night, driving through a hilly, wooded part of southern New England, I wondered about this. If not like an organism, what is it like, what is it most like? Then, satisfactorily for that moment, it came to me: it is most like a single cell.
>
> Lewis Thomas, *Lives of a Cell*.

The fields of cytology and histology have provided a plethora of morphological and histochemical characterizations of various cells in diverse organisms. The next step in such instances as outlined in the General Introduction is to attain a deeper understanding of cell dynamics involving characterization at a molecular level. For this reason, gene expression profiling has become a must in many fields. Organs, tissues and cells populations have become routinely sorted based on expression of reporter genes and morphology. The advent of microarrays allowed the simultaneous characterization of thousands of transcripts. Advances in sequencing technology have rendered possible characterization of gene expression without *a priori* knowledge of transcriptomics at an increasingly high resolution [174]. One of the new frontiers in molecular biology is the characterization of gene expression at the level of an individual cell. Although amplification of small quantities of nucleic acid has been possible for decades, the ability to analyze it with such precision is novel. The evo-devo field has not yet taken advantage of this new approach.

3.1.1 Why single cell analysis transcriptomics?

Single cell analysis may appear at first as an unnecessary complication. Why analyze individual cells containing minute quantities of nucleic acid when one could gather a set of isogenic cells expressing sufficient quantities of nucleic acid for analysis and examine those by simpler means? The problem lies within the heterogeneity of cells in any given population. Are the populations composed of truly identical cells? Gene expression profiles generated from cell populations of hypothetically identical cells can mask the presence of rare or small numbers of heterogenous cells. Examination of the gene expression from a pooled sample would be representative of the majority of the cells present, and hide minority populations. Such a "summation effect" has been observed even in prokaryotes and multicellular eukaryotes alike. "Persister" bacterial cells with distinct gene expression features are in a minority in normal conditions but in some cases can lead to drug resistant survival [175, 176]. Similarly, small numbers of dormant haemoatopoietic stem cells play an essential role during wound healing and regeneration [177]. Examination of *Xenopus* oocyte maturation via pooled gene expression studies could suggest that maturation-inducing hormone progesterone acts in a continuous fashion, although individual oocytes demonstrate an all-or-nothing response and that there is no "average" oocyte" [178]. Instead, there are two non-overlapping subpopulations of oocytes [176]. The relevance of the heterogeneity of cells has been of particular importance in cancer studies [179, 180]. Single cancer cells deriving from a clonal population display different dynamics and responses to treatment [181, 176]. Furthermore, low abundance mRNAs present in a minor population of cells may not be detected all together, resulting in a type II error [174]. Transcription factors are often expressed in low amounts and may represent such a class of transcripts. Moreover, one need not look necessarily for situations with cryptic populations of cells, as if two cell types are pooled and analyzed, the common gene expression pattern will be detected, whereas the individual profiles will be diluted. Increasing amounts of evidence indicate that it is the so-called "noise" or differences between cells that are the driving force of cell fate selection [182, 174, 182]. High resolution analysis also runs the risk of reaching the limit of stochastic technical replicability [176].

3.1.2 Obtaining a single cell RNA

Obtaining single cells Most single cell gene expression profiling have used low throughput methods for obtaining the cells of interest such as patch-clamp aspiration of cytoplasm, axoplasm or dendroplasm, picking individual cells using a micropipette [184, 185, 186, 187, 188]. Whereas Laser Capture Microdissection (LCM) which consists of placing an acetate film over tissue slice. Cells of interest can be peeled off the film in an automated fashion [189]. This approach however has the drawback of being quite time-consuming and labor-intensive. RNAs obtained in this fashion run the risk of being degraded during the procedure. Recently, considerable effort has been invested in microfluidic chip that can not only isolate individual cells, but on which single cell lysis, and cDNA synthesis can be combined [190]. The Lab-on-chip approach has raised high hopes in combining cell culture, morphological and nucleic acid characterization [191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201].

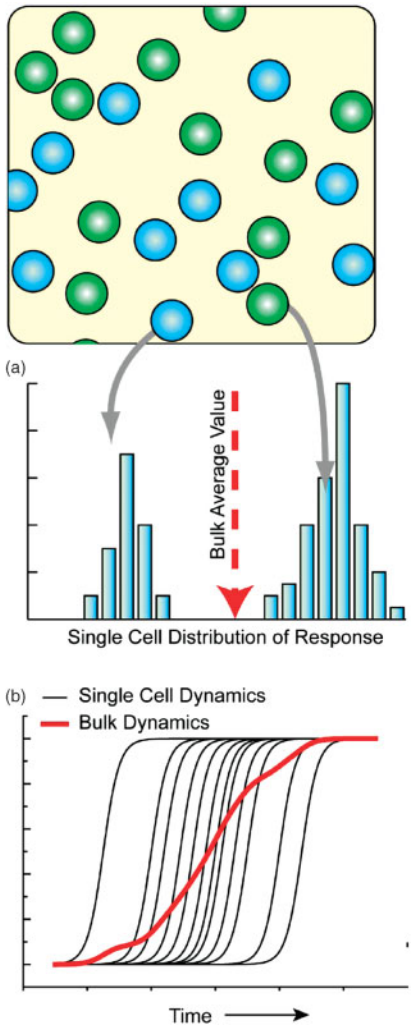


Figure 3.1: Reasons for studying single cell gene expression. Pooling of several cells can have several consequences: (a) due to population heterogeneity that may manifest itself in non-normal distributions (i.e. bimodal). (b) Bimodal responses may manifest themselves because of fast time-dependent changes in individual cells from one state to another, which vary in time amongst the population. Both the kinetics of the response and the average value can be misinterpreted using bulk techniques(Image and caption reproduced from [183].

Most commonly available RNA isolation protocols consist of numerous steps during which nucleic acid loss may occur. When dealing with small amounts of RNA the risk of loss may be deemed unacceptable. Most commercially available kits make use of chaotropic agents and proteases to inactivate RNases. However, these substances may inhibit subsequent reverse transcription and PCR. Hartshorn and colleagues devised a chaotropic solution optimized for single cell applications [202]. Most single cell protocols have however completely skipped the single cell isolation step, by simply lyzing the cell by heating at 65 to 70 $^{\circ}$C or freeze-thawing. A much more elaborate method was devised by Jena and colleagues who fractionated nuclear and cytoplasmic material using detergents that disrupted the cell membrane without affecting the nuclear membrane and then separated the nuclei by centrifugation [203].

Among the first flow cytometry experiments on live single cells based on fluorescent gene expression were performed on the *C.elegans* touch-receptor neurons [204]. This approach was later implemented in the study of olfactory, thermosensory neurons and GABAergic motoneurons [205, 206]. Sorting based on fluorescently labelled cells has been implemented in mammalian systems on a variety of cell populatins such as neural crest stem cells that were differentiating into Schwann cells [207], subpopopulations of the CNS using retrograte labeling of cortical neurons at different stages of development [208]. Sugino and colleagues manually sorted labeled populations of mammalian forebrain cell populations. The non-automated aspect allowed the authors to purify adult neurons and to isolate rare subtype of neurons [209]. Fluorescent reporter gene labeling is not the only labeling method that has been employed for the transcriptome characterization of small populations of cells. Marciano implemented terminal deoxyribonucleotidyl transferase-mediated biotinylated dUTP nicked end labeling (TUNEL) and caspase-3 immunohistochemistry to identify cells undergoing apoptosis in the CA3 and dentate gyrus regions of the hippocampus following traumatic brain injury. Using this method the authors could identify different stages of cell damage and apoptotic cell death [210]. Yet another approach to purifying mRNA from a genetically labeled cell population is RNA tagging. By FLAG-tagging the poly(A)-binding protein (PABP) expressed under a cell type specific promoter in which it binds to practically all polyadenylated mRNA. After fixation and cross-linking PABP to covalently link the mRNA the whole organism or tissue of interest is homogenized and the mRNA is purified by anti-FLAG affinity purification. This approach was implemented to analyze gene expression in ciliated sensory neurons in *C. elegans* [211] and the photoreceptors of *Drosophila* [212].

3.1.3 How much RNA is in a Single cell?

Indeed a single mammalian cell is assumed to carry 10-40 pg of total RNA, of which about 0.1- 1 pg mRNA corresponding to 10^4-10^6 transcript species, most of which are expressed below ten copies. Housekeeping and effector genes are expressed at higher levels of 10^2 -10^4 copies, whereas transcription factors are less abundant at $<$100 copies [213, 214, 215]. These figures are in stark contrast with the requirements of most gene expression profiling systems which require micrograms of nucleic acid or more, thus requiring either the collection and analysis of at least 50 000 cells [216] or the mRNA to be amplified over a million-fold [199].

3.1.4 Sample preparation

Tools for amplifying single-cell or small-population-of-cells-quantities of nucleic acid have been available for decades. However, the capacity to analyze them at a high resolution is new and at relatively low cost is new.

Reverse transcription Indeed, the discovery of the RNA dependent DNA polymerase or reverse transcriptase by Howard Temin and David Baltimore [217, 218]) dates back to 1970. Two years later, three independent groups working on globins and rabbit reticulocyte put to use Temin and Baltimore's enzyme with oligo(dT) primers to *in vitro* synthesize single stranded cDNA from mRNA templates [219, 220, 221]. These discoveries lead to the obtention of the first clones of complementary DNA (cDNA). Simultaneously methods allowing the cloning of DNA fragments into plasmids became available [222, 223, 224, 225, 226, 227]. cDNA cloning methods relied on poly(dA)-poly(dT) tailing method [228] [229, 230] Rougeon used oligo(dT) to prime synthesis of the first strand of cDNA, and added dT residues to the 3' end of the cDNA with terminal transferase. With an oligo(dT) tail on both sides of the cDNA population, a second strand could be synthesized using an oligod(A) primer. The duplex cDNA was then tailed with oligo(dC)[223]. Addition of poly(dT) tails to the ends of a plasmid vector and the use of these tails to prime the first cDNA strand synthesis from mRNA was another approach developed at this time[226]. Molecules containing the cDNA at each terminus, were treated with terminal transferase to generate dT tails and then annealed to a plasmid containing dA tails. The resulting double plasmid containing two single stranded cDNAs was then used to transform bacteria. The second cDNA strand was synthesized in the bacteria. Thanks to advances in reverse transcription and gel electrophoresis full-length cDNAs could be obtained [224, 231, 225]. The most commonly used method during this time to synthesize and subsequently clone full-length double stranded cDNAs was established by Maniatis [231, 224]. The latter made use of the the fact that the 3' terminus of the first strand of cDNA primed the second strand by reverse transcriptase [232, 233, 234]. Originally, the avian reverse transcriptase (ALV) consisting of two polypeptides: an RNA-dependent DNA synthase (RTase) and Integrase which catalyzes the endonucleolytic cleavage of DNA as well as cleavage of the DNA-RNA duplex and RNase H which proceeds to the exonucleolytic removal of rNTPs. The Murine RTase isolated from the Moloney strain of Murine Leukemia Virus cannot degrade RNA in the RNA-DNA duplex as efficiently and lacks the endonuclease activity [235]. The Superscript reverse transcriptase was originally a genetically engineered version of the murine enzyme that carries mutations that reduce RNase H activity without affecting DNA polymerase activity [236, 237]. A variety of primer sets have been used including oligo(dT)$_{12-18nt}$ which anneal to the poly(A) tract at the 3' terminus of the mRNAs, primer adapters containing and oligo(dT) tract and a restriction site at the 5' terminus or a target sequence which can be later used for amplification [238, 239, 240]. Priming the first strand synthesis off of an oligo(dT) tract located in a plasmid was commonly used in the 1980s, however this approach was abandoned due to inefficiency [241, 242]. Random primers are still commonly used, although full length clones are difficult to obtain using this method. Throughout the 1970s and 1980s second strand synthesis was produced using self-priming. This method had the disad-

vantage of resulting in loss or sequence rearrangement at the 5' terminus of the mRNA due to the Nuclease S1 cleavage of the hairpin structure [243]. Second strand synthesis catalyzed by DNA Polymerase I and E.Coli RNase H which was shown to be less destructive. The advent of polymerase chain reaction (PCR) allowed cloning based on minute amounts of mRNA [244]. SMART technology made use of the MMLV RTase's capacity for template switching. On one hand this method had the advantage allowing the amplification of transcripts present in single copies, and on the other hand of inducing a bias towards shorter transcripts. Eberwine and colleagues used the self-priming RT method in the study RNA isolated from patch pipette aspirated cytoplasm after electrophysiological studies. Amounts obtained corresponded to far less than the amount of RNA from a single cell. Although, not quantitative, this method permitted exploratory studies yielding ground-breaking information about the presence of numerous species of transcripts in neurites [245].

The reverse transcription method can be modulated to produce longer or shorter transcripts based on the time taken. Studies which do not require full-length cDNA, often used rapid reverse transcription methods. One overlooked aspect of reverse transcription is the efficiency of the reaction due to the relative amounts of reagents used. Few, single cell analysis papers have examined the reverse transcription efficiency. Tougan and colleagues pointed out that based on Michaelis-Menton enzyme kinetics, reverse transcription reactions in which the enzyme is present in excess as compared to the single cell quantities of template should be expected to proceed quite slowly and that addition of unspecific template could speed up the the reaction. The authors added a 200 nucleotide long RNA species dubbed "Chum RNA" of known length and sequence to their RT reactions and observed an increase in total RNA efficiency[246].

Several types of reverse transcriptases are currently commercially available for the preparation of cDNAs. Invitrogen's SuperScript III (Invitrogen) is most widely used for this purpose and can generate full-length cDNAs of up to 10 kilobases (kb). Bengtsson and Taniguchi compared a set of ten commercially available reverse transcriptase enzymes (TaKaRa One step RNA PCR Kit(TaKaRa), BEST RNA PCR Kit (TaKaRa), DNA Synthesis Kit (TaKaRa), Super Script III (Invitrogen), ThermoScript RT (Invitrogen), MiScript RT(QIAGEN), Advantage RT (Clontech), PrimeScript RT (Clontech), ReverTra Ace-alpha (TOYOBO) and Transcriptor cDNA Synthesis Kit (Roche)) and observed that SuperScript III (Invitrogen), ThermoScript RT (Invitrogen),Advantage RT (Clontech)and Transcriptor cDNA Synthesis Kit (Roche) performed best [247].

Single cell amplification Once the single cell RNA has been successfully reverse transcribed, the amount of single cell cDNA remains limiting for further analysis. Individual gene-specific qRT-PCR can be carried out on a few selected genes of interest. Multiplex PCR increased the number of genes which could be studied simultaneously. However, full transcriptome analysis requires "global" RNA amplification. In numerous cases protocols established for the amplification of mRNAs derived from single cells were developed by using dilutions of total RNA, but were seemingly less successful using real single cells. One likely reason for this discrepancy may be that pipetting errors exclude precise quantification of mRNA copy numbers [249, 246]. The main approaches developed long before the microarray era are used for this purpose. On

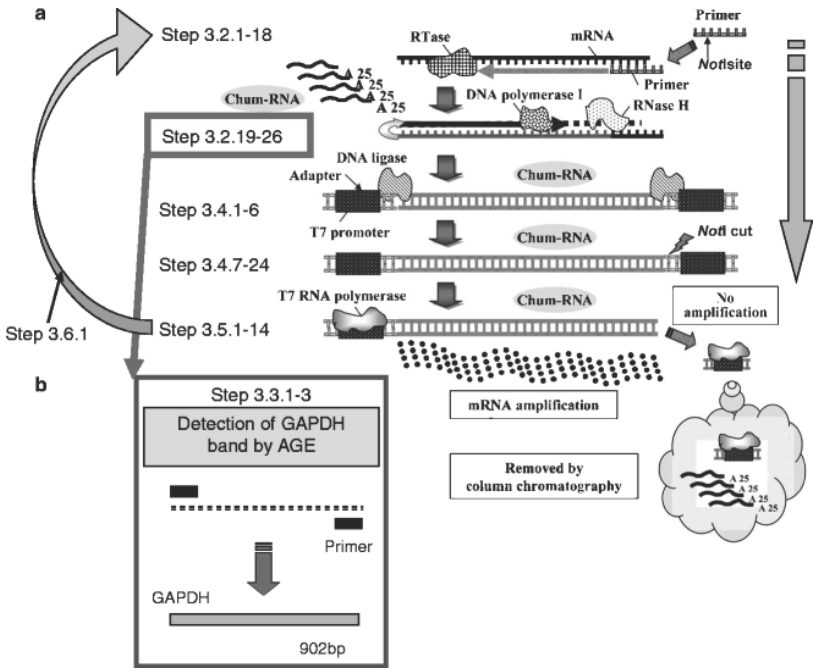


Figure 3.2: Schematic representation of the procedure used for the preparation of a cDNA library from a single-cell-derived amount of mRNA using chum-RNA. (a) Illustration of the procedure used for a single round of chum-RNA amplification. (b) Illustration of the procedure used for the preparation of a single-cell cDNA library after four rounds of chum-RNA amplification(Image and text from [248].

one hand Van Gelder devised a strategy to linearly amplify mRNA from small amounts of starting material for studies of gene expression in the brain. This method is known as the "Eberwine method". It consists of the reverse transcription of mRNA using an oligo(dT) primer containing a T7 RNA polymerase promoter site (Fig. 3.3. After second strand synthesis, antisense RNA is transcribed *in vitro* by T7 polymerase. This procedure can be repeated several times, and further increase the yield. The efficiency range of two rounds of linear amplification is approximately $10^3$to 10^5-fold. The RNA polymerase is thought to not be affected by template sequences or the concentration of template and therefore there should in principle be no differential amplification of different mRNA species expressed at different frequencies [98]. In non-microarray studies this method was used at single cell levels [250, 251, 252, 253, 254].

An Eberwine-based approach followed by gene-specific PCR was used to analyze the gene expression of different dopamine receptors in different cell populations of the striatal projection neurons [255]. The aRNA-based amplification confirmed by RT-PCR indicated, that D1-class and D2-class receptors were co-expressed striato-nigral neurons and that approximately 50% of striatonigral neurons D3 or D4 in addition to D1 receptor mRNA [256, 257] Among many pioneering efforts in this direction several additional technically difficult protocols were developed by the Eberwine group, such as a procedure that they called "*In situ* transcription" to localize and enrich transcripts at a single cell level. DNA primers were annealed to the RNA in a fixed tissue section. After washing away the unhybridized, cDNA synthesis was carried out by addition of reverse transcriptase and radioactive dNTPs. Unincorporated nucleotides were washed away. In this way cDNA of a gene of interest could be enriched and its localization determined. In this way localized gene expression profiling could be performed [257]. Another approach consisted of injecting cells with DNA polymerase and performing the reverse transcription and amplification within the cell of interest [258].

The "Eberwine" protocol suffers from a 3' bias due to oligo(dT) priming. An alternative strategy was implemented by Wang, taking advantage of template switching effect of reverse transcriptases which add additional non-template based cytosine to the 3' end of the cDNA. The reaction includes primers containing an oligo(dG) sequence at the 3' end which will base pair with a polyC stretch. The reverse transcriptase switches template and continues replicated the defined sequence of the annealed primer (Fig. 3.3. Linear amplification can be accomplished without passing through an RNA step. Single primer isothermal amplification (SPIA) for micoarray experiments utilizes a DNA polymerase [259]. Resulting cDNA generated by this method is less prone to cross-hybridization on microarrays than the frequently used cRNA [260].

PCR based methods on one hand have the advantage of providing large yields. On the other hand, due to the exponential amplification, the original relative abundance of the endogenous transcripts may not be faithfully represented. RNAs present at high frequencies can outcompete less abundant species for the primers. Furthermore, there is a bias towards shorter and more GC rich species. Amplification using aRNA increases the amount of cDNA in a linear fashion, permitting better representation of relative abundances of RNAs in the initial sample (Fig. 3.3). An oligo(dT) primer containing a T7 RNA polymerase promoter is used for the reverse transcription. The cDNA is then *in vitro* transcribed back to produce cRNA. Yields can reach 1000x to 5000x after two rounds of amplification. The most frequently used protocol for single cell RNA ampli-

fication was developed by Brady and Iscove [261]. In order to avoid nucleic acid loss, cells were lysed directly in reverse transcription (RT) buffer containing non-ionic detergent and the cDNA synthesized is initialized immediately. By limiting the duration of the reverse transcription reaction and the concentration of primers the length of the cDNA is controlled, avoiding a bias against longer length transcripts. Since PCR reactions are more efficient for shorter sequences, amplification of full-length cDNA would result in disproportionate amplification of smaller cDNAs(Fig. 3.3). This bias can be avoided by limiting the length of the initial cDNA strand to around 100-700 bases regardless of the size of the original RNA template. cDNA is then tailed with polyA at the 5' end using terminal transferase and amplified using a primer that includes a 3' stretch of oligo(dT)[262]. Several variations of this protocol have been developed and used with varying efficiency to amplify picograms of RNA [263, 264]

As the Eberwine protocol is labor-intensive and time consuming the two-step amplification is rarely described in literature. As the amplification efficiency of PCR based methods is higher and has been claimed to reproduce transcript heterogeneity more faithfully, several modifications and adjustments of the original Brady-Iscove method have gained popularity instead. For this reason several other methods continue to be developed. For example, another exponential amplification approach is SMART PCR which uses adapter sequences that are added both ends of the cDNA during synthesis for subsequent amplification [265]. The manufacturer's website recommends this method using at least 100ng of starting material. Attempts to use SMART amplification with picograms resulted in a high rate of false positive and false negatives using microarrays [266].

Terminal continuation (TC) RNA amplification is another method that has been implemented for the study of small cell populations. This method involves the attachment of a primer of known sequence to the 3' region of the first strand cDNA in addition to oligodT priming, therefore allowing the ss cDNA synthesis by annealing a second primer complementary to that attached. This methods avoids using the S1 nucelase digestion step and stem loop formation. Furthermore, TC RNA transcription can be initiated using a promoter sequence attached to either the 3' or 5' primers, allowing discrimination between the orientation of transcripts ('antisense' or 'sense') [267, 268]. Whereas in the Three-Prime End Amplification (TPEA) approach, generic upstream primers are annealed to a random sequence that occurs every few hundred bases of mRNA sequenc [188]. Furthermore, numerous commercial IVT kits are available for "preamplifying" mRNA samples for microarray analysis. The comparative analysis of yield and efficiency of these kits is contradictory. Most kits however require nanogram quantities of starting material. Sigma's WTA uses semi-random primers for reverse transcription followed by PCR for cDNA amplification to generate micrograms of nucleic acid. A recent communication by Sigma attested that primer dilution in the reverse transcription step results in increase in relative mRNA frequencies from small quantities of starting material. This amplification method does not allow the user to distinguish between the original genomic DNA strand from which the mRNA was transcribed. The WTA method uses primers on both side of the cDNA and does not allow the directional determination. Moreover, due to the lack of oligo(dT) priming mRNA are not selected, resulting in a large amount of ribosomal RNA amplification [269].

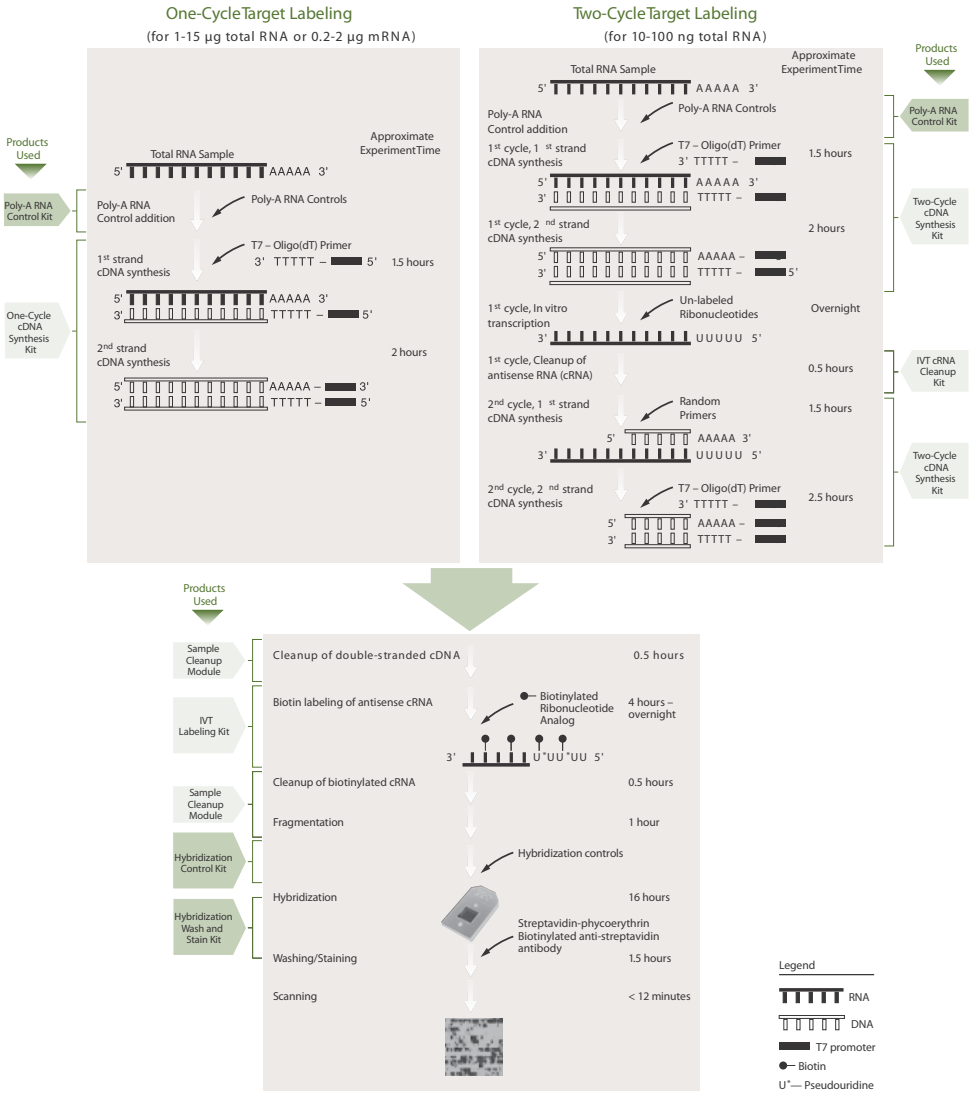


Figure 3.3: Linear amplification of small amounts of RNA for Affymetrix chip hybridization using one (top left panel) or two cycle (top right panel) linear amplification before hybridization to Genechip DNA microarray(bottom panel) Image reproduced from GeneChip Expression Analysis Technical Manual p. 24

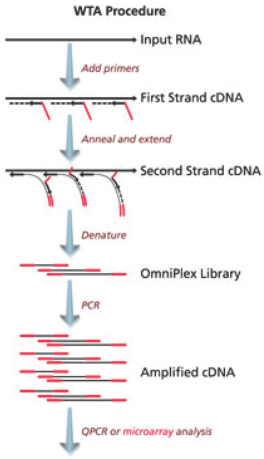


Figure 3.4: Sigma's Whole Transcriptome Amplification Kit Procedure. Total RNA is heat denatured and reverse transcription with a strand-displacing reverse transcriptase primed with quasi-random non-self complementary 3' primer and a constant non-self complementary 5' primer generates a double stranded cDNA library which is then amplified using PCR

Expression profiling The techniques used to amplify nucleic acid are closely linked to those used to then analyze the resulting material. Prior to the advent of genome sequencing and the wide use of microarrays, PCR based techniques such as differential displays have been utilized for subcellular amounts of nucleic acid present hippocampal neurites, demonstrating an unexpectedly large number of mRNAs are present in the neurites of hippocampal cells in culture[270]. Standard gene expression profiling methods such as SAGE have been scaled down to the single cell level. microSAGE has been used to study the expression profiles of human mesenchymal stem cells [186] and rat brain "punches" [271, 272]. SAGE involves sequencing long DNA concatemers of a series of short pair tags cut from reverse transcribed cDNA using class II restriction endonucleases amplified by PCR. This method is considered better at demonstrating relative and absolute abundance of mRNA species present in the sample analyzed. Microarrays allowed the simultaneous examination of thousands of genes. This method however requires prior knowledge of the transcripts expressed to be present on the chip. Standard Affymetrix protocols utilize linear one or two-step T7 polymerase mediated amplification. Microarrays have been successfully adapted for single cell expression studies [167, 273, 274]. There are however a number of drawbacks and limitations to using microarrays as the latter only contain probes only for known genes. This requires the a priori knowledge of a complete genome and possession of an inbred line of animals or a clonal population of cells to study their gene expression in such a way that polymorphisms would not interfere with the analysis. Furthermore, the discovery of unknown mRNA cannot be carried out. Alternative splicing can only be studied if

specific probes spanning different exons have been incorporated on the chip [269]. To facilitate comparison of microarray experiments spike-in controls have been suggested as mandatory [275]. Despite the popularity of microarray platforms, their sensitivity lags behind that of quantitative qPCR.

For the moment, RT-PCR-based methods remain the most high throughput method providing the most consequent yield in terms of material. Digital PCR single cell characterization performed by Warren and colleagues attests to this. Using microfluidic devices Warren and colleagues were capable of performing a large number of individual reaction. Bengtsson and colleagues using a traditional benchtop approach could not detect transcripts expressed in 10-20 copies per cell, whereas Warren and colleagues claimed to be able to count individual mRNAs.

The main difficulty in gene expression studies at the single cell level, is their static nature. Whereas, mRNA concentrations are the results of a delicate balance between transcription and degradation. By taking samples at different time points, it could be possible to estimate the half-lives of different RNA species and turnover. Advances have been made in quantifying actively translated mRNAs, although this field is only at its beginning[276].

The next "frontier" so to speak is sequencing of samples directly. Sequencing-based methods are rapidly replacing microarray-based gene expression platforms and have allowed whole genome sequencing. The different methods combine different sample preparation methods and sequencing approaches. The automated Sanger methods implemented over the past four decades termed "first-generation" are rapidly becoming replaced by newer methods referred to as "next-generation" sequencing or NGS. Templates usually in the lower microgram range are generally prepared by fragmentation and subsequently immobilized a solid surface in order to perform numerous sequencing reactions simultaneously. Emulsion PCR and solid-phase amplification are commonly applied in systems that do not allow single molecule sequencing and involve PCR reactions which can involve the drawbacks listed above (section 3.1.4, reviewed in [277]). This may lead to the further disproportional representation of relative transcript ratios. Single molecule sequencing approaches such as Helicos may obliviate this step, and allow more accurate profiling and expression level differences and yield information about splice-isoforms. For the moment such approached still require several hundred cells and can detect only 15-25% of expressed mRNAs [277, 276, 278].

Criteria for sample choice of method The general consensus in terms of small target sample amplification is the unavoidable "decrease in correlation coefficients and concordance when evaluating diminishing input RNA amounts" [279, 280, 281]. When comparing 200 ng, 10 ng and 2 ng with 10 μg - discrepancies increased with lower sample sizes [280]. Goley and colleagues performed serial dilution of RNA subjected to amplification. The resulting amplification products showed increased differences as compared to unamplified RNA [282]. This loss of linearity in T7 based amplification protocols has been considerably discussed by numerous authors [209, 283]. Therefore, the loss of transcripts is an important aspect in choosing a sample preparation method. Cells sequenced to date have been claimed to possess 10^4-10^6 transcript species. The chance of loosing one of them during the sample preparation procedure may appear

quite high. Some authors have utilized pooled cell samples to ensure detection of the less abundant transcripts. Using a spiking experiment 1, 2, 10, and 100 copies of a exogenous bacterial RNA were spiked into lysed solutions of single olfactory neurons [284]. After amplification the authors could detect the 100 and 10 copies, however the 1 and 2 copy spikes were not consistently present. Such controls could ensure the representation of even rare mRNAs. Rare transcripts down to 0.7 copies per cell can be detected in single hippocampal CA1 neurons as confirmed by qRT-PCR [285]. Nygaard and colleagues estimated the impact of linear T7-based amplification on the preservation of gene expression ratios as compared to non-amplified material. The authors found that 10% of the genes investigated showed significantly different expression ratios. The amplified sample gave a better signal-to-noise ratio and allowed detection of more less abundant genes than unamplified RNAs. These results corroborate several RNA amplification studies [283]. The commercially available Transplex system is also based on an exponential amplification. An independent evaluation of this method showed good comparisons of amplified and non-amplified RNA using 12 to 300 ng starting material [260]. This method was however shown to be less faithful with starting template amounts below 10ng. Gonzalez-Roca however demonstrated that by diluting primers 8-fold before reverse transcription began, relative ratios could be restored even with single-cell amounts of starting material. qRT-PCR is considered to be the most accurate to quantify the number cDNA copy numbers. The reliability of these measurements, however, depends on the accuracy of RNA extraction, cDNA preparation and pre-amplification steps [202].

Roca-Gonzalez and colleagues proposed four criteria to evaluate whether a method can be considered accurate for gene expression profiling of small amounts of transcript:

1. ”diluted samples should provide comparable results as the concentrated samples do when standard methods are used
2. diluted samples should provide results by profiling comparable to qPCR
3. technical replicates from the same RNA preparation should provide small differences in results
4. small cell populations should provide results similar to bigger populations” [269]

The numerous modifications of the Brady-Iscove protocol have been used with varying success [262, 286, 284, 287]. Among the most popular protocols currently available is the one proposed by Kurimoto and colleagues [167, 288]. As pointed out by Roca-Gonzalez and colleagues this method (adapted by Tang for sequencing) do not fulfill criteria 1 and 4. Furthermore, the sequencing and qPCR expression data comparison(criterion 2) excludes 70% of the measurements performed and use only the top 100 most abundant genes based, implying that the remaining measurements may not be as reliable. Roca-Gonzalez and colleagues claimed that if one were to ignore qPCR-based quantification confirmation experiment the results of the sequencing present large amounts of false positives and false negatives [269].

Hence, a paradox becomes apparent. The main criterion used to determine whether an experiment has been successful is replicability. Yet single-cell experiments by definition intend to characterize specific features of individual cells. The more replicates an

experimenter performs, the more stochastic gene expression within a single cell-type will become apparent, thereby hampering detection of cell type specific transcripts [289].

3.1.5 The paradox

Quaranta and Garbett track down this paradox to ”the dominance of deterministic thought in cell biology” [290]. They argue that heterogeneity and cell-to-cell variability are a ”fundamental property of living systems” and that variability will inevitably arise from processes such as transcription and translation. They argue that over the past 50 years, ”noise” which they define as ”an unresolved mix of experimental or biological fluctuation as well as meaningful biological variability” has become viewed as ”nuisance factor” by biologists, who instead of accepting the inherent variability of studied processes and taking it into account, tend to find means to eliminate it by statistical methods. Indeed, already in 1957, Novick & Weiner had demonstrated the variability of β -galactosidase expression in bacterial cells using serial dilutions down to individual cells [291]. Their results were later corroborated by in single mammalian cells [292].

Noise can be subdivided into three distinct categories: stochastic, intrinsic and extrinsic. Noise resulting from manipulation can further induce variability in the sample preparation process.

Stochastic noise It is however important to bear in mind that the concept of ”noise” or ”stochasticity” actually means ”random”, that is occurring in the absence of a ”deterministic” causal event, and therefore inherently unpredictable. Perceived randomness is not the same as true randomness. Gene expression is a multi-step process sufficiently complex for deterministic causation to not be easily determined or not be determined at all. However, the lack of information allowing this kind of conclusion is not sufficient to consider the events truly random. Seemingly random behavior can be observed in a deterministic setting as in the ”butterfly effect” and hence ”a multi-step processes with deterministic causation [...] can be so complicated as to be practically unpredictable” [293].There is a fundamental problem in empirically separating stochasticity from determinism.

Intrinsic variability Intrinsic variability results from the inherently ”blurry” nature of the processes evaluated. Even in a hypothetically constant and homogeneous intracellular environment the complexity incurred from conformational changes in DNA, repressers and transcription factors randomly binding and disengaging from cis-regulatory elements, and the complexity of transcription and translation, ”would produce random fluctuations in the number of mRNAs and proteins per cell, constituting ”noise” that cells must either exploit, learn to live with, or overcome using various noise-suppression mechanisms”. Gene expression indeed been proposed to take place not in a continuous fashion but in ”burst-like” stochastic events. First demonstrated quantitatively in *Bacillus subtilis* using GFP reporters, observed variability was interpreted as result of low abundance mRNA fluctuations and translation bursts [294]. Studies

in *E.coli* using correlations between dual fluorescent reporters interpreted the so-called "intrinsic noise" as a consequence of low abundance mRNA fluctuations [295, 296]. Golding and colleagues observed bursts in *E.Coli*, proposing that the latter correspond to transcription factor binding or unbinding, DNA conformational changes and sigma factor [72].

Variability in reporter expression was also observed in eukaryotes. Based on an original study in *Saccharomyces cerevisiae* the so-called "intrinsic noise" was attributed to chromatin remodeling which resulted in "quantal transcription bursts" [297]. A subsequent study in the same organism reinforced the idea of transcriptional bursts [298] where alteration of chromatin-remodeling enzymes resulted in changes in stochastic gene expression. Raj and colleagues showed that the genomic position (and therefore chromatin) affected covariation bursting of multiple genes [299].

This discovery has furthermore led to the proposal of several mathematical models. Peccoud and Ycart proposed that a gene randomly transitions from transcriptionally active to inactive states. Based on their data, Raj and colleagues hypothesized that the amount of transcription factor could modulate burst sizes without affecting burst frequency [300]. These models have been shown to apply to some experimental data, but not bimodal mRNA distributions resulting from "long transcriptional bursts". Pedraza and Paulsson have criticized these models because they assume that gene activation and disactivation events follow a Poisson distribution [299].

Extrinsic noise Extrinsic or global noise is not caused by molecular processes within the cells, but are associated with individual molecular processes within the cell. Changes in the microenvironment of cells can have dramatic effects on processes such as differentiation of hematopoietic stem cells. Other examples could be cell-to-cell interaction and receptor ligand biding, contacts with the extracellular matrix and various environmental stress [183]. For this reason increasing emphasis is being put on controlling the cellular microenvironments using microfluidic setups.

Distinguishing the indistinguishable The different types of variability that arise are therefore difficult to dissect *a priori*. Indeed in absence of further information on cellular processes, distinguishing between shear stochasticity, intrinsic, extrinsic noise and manipulation induced error is a daunting task. Increasingly, sampling of numerous single cells and carrying out comparisons is considered the way forward. Indeed, Janes and colleagues used a method they called stochastic profiling. By repeated random sampling of very-small cell populations from laser-capture microdissection and amplification using the Brady and Iscove protocol. Out of the 4,557 transcripts expressed in cultured human epithelial cells, 547 showed strong cell-to-cell expression differences [182]. The authors argued that "molecular techniques focusing on a single cell bring with them sufficient experimental error to interfere with the very measurement they are used to take; experimental and biological noise drowns meaningful heterogeneities". Using stochastic sampling, instead, "meaningful heterogeneities" can be observed [290].

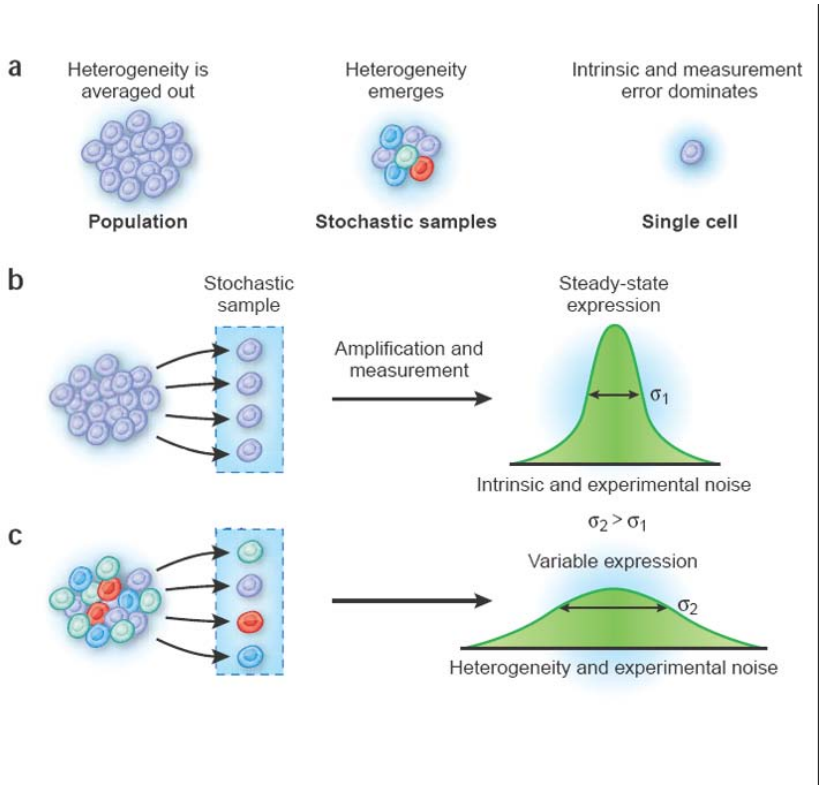


Figure 3.5: Caption from [290]: (a) In populations, any single-cell heterogeneity is lost, and a mean of the population is observed. With smaller samples, taken multiple times, heterogeneity of cells emerges. In a single-cell sample, measurement error can easily dominate and result in false positives when searching for heterogeneity. (b) A homogeneously expressed gene, when stochastically sampled, will have intrinsic and experimental noise and serves as a reference for these types of noise. (c) A heterogeneously expressed gene will have a wider variance when compared with the reference in b. Such a comparison is not possible in single-cell measurements.

The implications of variability The implications of the variability have deep implications in our way of performing and perceiving science. On a practical side, "housekeeping" genes traditionally used as references in gene expression studies, previously considered invariable and of insignificant biological importance, may no longer be used as such [301]. Cell types originally considered functionally quasi-identical such as *Drosophila* R1-R6 photoreceptors, have been shown to differ considerably in terms of gene expression [302] and hematopoietic progenitor cells expressing different quantities of Sca-1 can give rise to different cell lineages[303, 176]. Moreover, organisms have traditionally been divided into those whose developmental choices are "tightly regulated" by molecular determinants, such as flies or *C.elegans* and those whose regulative development choices are based on stochastic events such mammals. In light of the large variability observed between individual cells, these categories may be revised. Perhaps, there is a continuum between the stochastic processes and ordered "regulated" processes.

3.2 Results

In order to perform molecular cell characterization, cells need to be successfully and faithfully extracted from whole organisms. A dissociation procedure (see Chapter 2) is the first step. Isolation of the cell of interest from the resulting dissociated tissue or organism is the next step. However, although cells *in toto* can be identified through their position within the tissue or organism and via their respective morphology, dissociated cells *in vitro* lack the positional clues necessary to their identification and can loose characteristic morphology without the pressure exerted by surrounding cells and body. For this reason, a morphological characterization of *Platynereis* cells was carried out by stage, in order to determine whether any cell types could be distinguished on a purely morphological or behavioral basis. Based on this information, a pilot molecular study was initiated on selected easily identifiable cell types. Different conditions for cell isolation and nucleic acid extraction were tested to determine which conditions were optimal for the isolation of RNA from individual cells.

Platynereis morphological characterization of blastomeres and pre-10hpf larvae-derived cells

Platynereis dumerilii displays a stereotypical developmental pattern. During early stages of embryonic cleavage, all the cells produced are predetermined and have been described in great detail by Dorrensteijn and colleagues [159]. A brief morphological characterization has in part already been discussed in Chapter 2 in regard to determining the quality of *Platynereis* blastomeres obtained from the dechorionation procedure utilizing sodium thioglycolate and Pronase. To study the cell behavioral features of blastomeric cells, dechorionated embryos were studied in two ways.

First, dechorionated individual embryos were observed at regular intervals using a Zeiss Axiophot with a motorized stage. Secondarily, a small screen was performed in which 2 to 4-cell stage dechorionated embryos placed in individual wells of a a 96-well plate in SFNSW for 24 hours.

Development of dechorionated *Platynereis* embryos In 1949, Donald Paul Costello claimed that he could follow the development of dechorionated *Nereis limbata* embryos[304]. Costello used the alkali NaCl and $CaCl_2$ method (tested in Chapter 2) for the denudation of larvae. Costello claimed that he could reproduce "normal" *Nereis* development in the absence of a chorion. Moreover, Costello claimed that he could deduce the cell fate of individual blastomeres and partial embryos based on his experiments in rearing dechorionated embryos. In my hands this experiment was not reproducible in *Platynereis dumerilii*. Control embryos dechorionated at 2 or 4-cell stage did not develop correctly past 6-7 hours after fertilization. Results rather resembled those observed by Marina Dan-Sohkawa on dechorionated *Asterina pectinifera*.

Cells resulting from the first set of cleavages (similarly to those of *Asterina pectinifera*) demonstrated weak adhesion one to another. Indeed as described in Chapter 2, cells could be easily detached one from each other after cytokinesis was complete by simply pipetting blastomeres up and down using a capillary, whereas Dan-Sohkawa observed independent behavior of cells when subjected to a weak water current. Cells began to adhere one to another only several hours later, approximately 4.5-5 hours post fertilization after the 9th or 10th cell cleavage (similar events took place after the 8th cell cleavage in *Asterina pectinifera*) . As proposed by Dan-Sohkawa adherence between cells may indeed correlate with the appearance of pseudopodia or thin cellular projections (See Fig. 2.12 white arrows). Dissociated cells corresponding to approximately post 20 cell stage began to aggregate into large cell masses composed of single or double cell layers. In *Asterina pectinifera* cells aligned themselves in a single sheet and the free edges of the base of the cluster curled upward to form a hollow blastula. Blastula formation was not observed in *Platynereis*.

Several explanations could be proposed to explain why Costello's observations could not be reproduced in the present study. Costello performed his experiments on an organism he called *Nereis limbata*[304, 148]. The present classification of annelids names this organism *Alitta succinea* (Leuckart, 1847). Although numerous similarities in the development of *Alitta succinea* and *Platynereis* have been described, it is not inconceivable that differences in terms of cell adhesion and embryonic development may exist. Moreover, denudation as performed by Costello was not successful in *Platynereis* (see Chapter 2 and Table 2.2.2, Table 2.2.2 and Fig. 3.7) [305, 306, 148]. Precise measurement values were not available and could explain the difficulty to reproduce denudation procedure utilized by Costello. Finally, Costello's data did not enjoy any follow up studies, indicating a possible difficulty in reproducibility. The difference between the observations performed by Dan-Sohkawa who did indeed observe "normal" larval development of *Asterina pectinifera* and the present observations may be explained in several ways. Starfish embryos utilize a equal holoblastic cleavage type. The first cleavage furrow passes through both animal and vegetal poles, and cuts the egg into two equal sized blastomeres. The second cleavage is at right angles to the first. The third pair of cleavages are horizontal and produce eight blastomeres. —Platynereis embryos follow a spiralian cleavage. Briefly, the first two cell divisions result in the production of four macromeres which correspond to "quadrants" of the embryo. Both these cleavages are oriented in planes that occur at right angles parallel to the animal-vegetal axis of the zygote. After each cleavage cycle, the macromeres give rise to "quartets" of smaller micromeres at the animal pole. Furthermore, *Platynereis*

embryos practice unequal cleavage producing different sized cells as of the first cell division. Thus unlike the early *Asterina pectinifera* embryo the *Platynereis* blastomeres asymmetricly inherit unequal cytoplasmic and membrane regions, which may confer specific properties to each cell from very early stages of development.

After over 24 hours in a 96 well plate at room temperature, true trochophore larva were never observed (n=384). However, ciliated cells and pigmented cells were clearly distinguishable in some samples(n=9). Cells remained quite large which is characteristic for early blastomeres. Engstrom had observed that *Urechis* dechorionated embryos were more elongated. This was not observed in *Platynereis*. Engstrom had also observed double-embryos. This was not observed in the present study either. Unlike the Echiuroid fertilization membrane, the *Platynereis* chorion is necessary for correct development. The chorion is not re-secreted or reformed after removal. Its function amongst others is that of protection of the embryo from external stresses including osmotic shock but also to keep the cells tightly bound together, since little if any adhesion is observed between blastomeres.

Time lapse recording of dechorionated embryonic development of *Platynereis* development The time lapse observation of dechorionated embyonic development was performed starting at 2-cell or 4-cell stage. Similarly to the observations made by Costello and Engstrom, embryonic divisions as compared to those of non-dechorionated embryos were considerably behind [148, 307]. Engstrom observed a delay of 5-10 minutes in Echiuroid *Urechis* "membraneless" worm development. The cell divisions observed in the present investigations took place approximately every 30-40 minutes (Fig. 3.6). In *Platynereis* unlike in Echiuran, membraneless embryo development, the cleavage planes were not identical. This can however also be attributed to pressure from the coverslip on the sample, which unlike in a chorionated embryo whose cells are only slightly compressed yet held in place by the chorion, can result in cell movement in the direction where there is space. Unlike Engstrom, "gastrulation" or rather internal cell movement was not observed. Costello claimed that he could trace the developmental fields of each of the early blastomere segments of *Nereis limbata*, often observing practically fully developed larvae [304].

Cell lineage tracing was attempted using the timelapse recordings performed on dechorionated embryos. *In vivo*-style cell division was never observed (Fig. 3.6). Among the striking details observed were the failed attempts at cell division. When cells began to divide, they would move their cytoplasm and elongate before undergoing cytokinesis. In numerous cases, the larger cells were observed to extend to the outside of the embryo cluster and elongate moving away from the main cell mass. These cells would undergo cytoplasmic movements resembling those undergone by dividing cells, and suddenly shrink back to a spherical shape and return to the main cell mass. This phenomenon was observed quite often, and in part these cells may be responsible for the inaccurate cell lineage (Fig. 3.7).

Figure 3.6 *(following page)*: Examples of cell lineage tracing on dechorionated embryos. Accurate lineage and timing of cell division was not observed using time lapse microscopy for up to 430 minutes (A). Cells could be observed for prolonged periods of time, and lineage traced, however embryos never "caught up" with their non-dechorionated siblings. In some instances, all observed cells were seen to divide (B), ceasing synchronous division after less than an hour. In other cases, the first blastomeres did not divide such as AB in (C). In a third group of embryos, blastomeres could either not be traced back or were observed to divide more than once, thus breaking with a spiralian pattern of cell division (Numbers on Y-axis correspond to time in minutes)

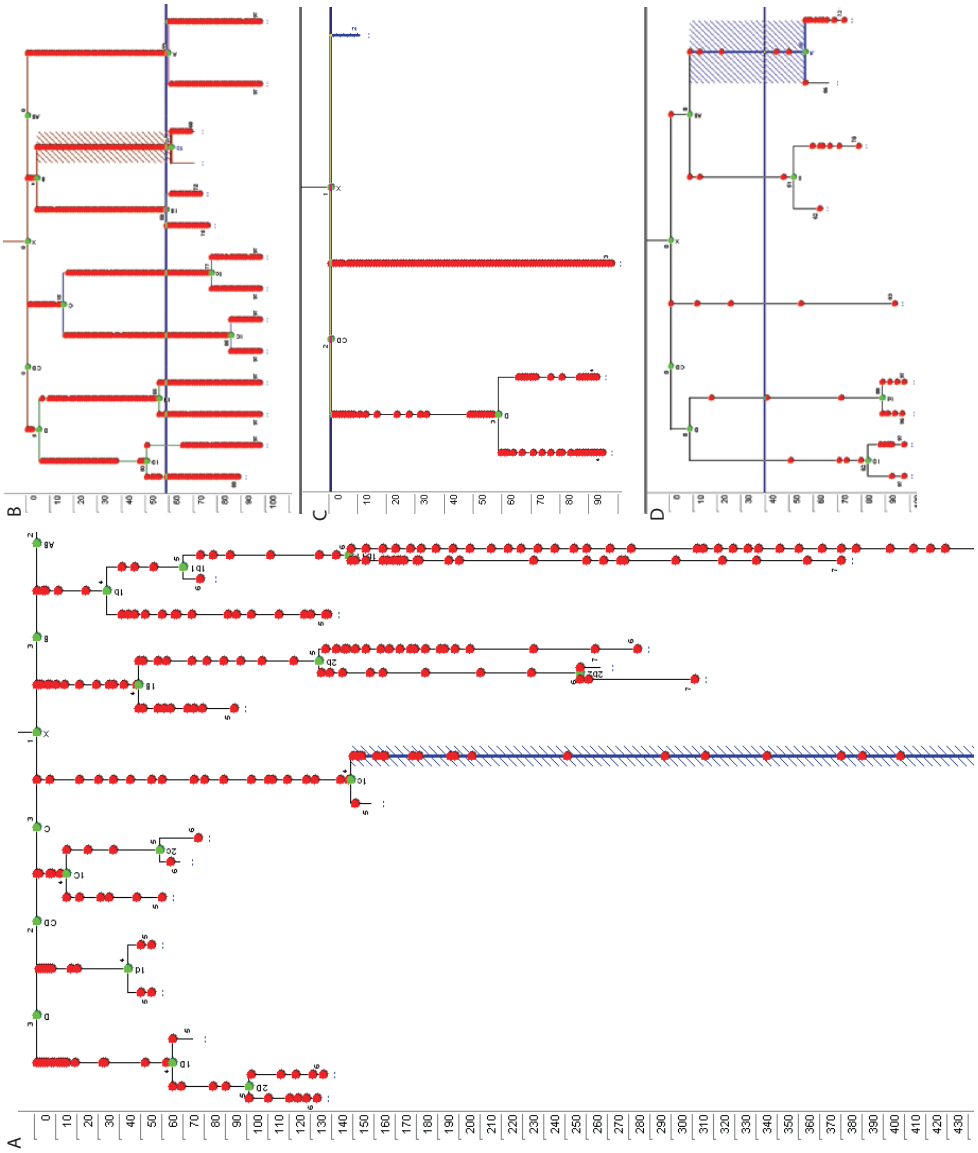

A
B
C
D

Figure 3.7: Dechorionated embryo development. (A)Large cell observed to move away from the rest of the embryo(black arrow), elongate, fail to divide and return to spherical shape. (B) Two larvae observed. Left larva did not go through cell division for the period of time examined, whereas in right embryo large cell is observed to move up, elongate, fail to divide and then to reacquire spherical morphology and return to its initial position. A second large cell observed to emerge (far right) from underneath the embryo is observed to exhibit the same behavior.

3.2.1 Morphology of cells derived from pre-10hpf larvae

Cells derived from larvae younger than 10 hours are large, round and transparent varying in size between 20 to 50 μm. The smaller cells aggregate easily but neither small nor larger cells adhere well to plastic or glass, indicating an absence of expression of adhesion molecules necessary for substrate adhesion. A nucleus can be easily distinguished (Fig. 3.8). Also, smaller dark granular structures are observed(Fig. 3.8A and Dii). The only cell types that can be distinguished from other cells are macromeres. Each embryo contains 4 macromeres (Fig. 3.8C Black arrows). These cells contain the characteristic lipid droplet (Fig 3.8D). These cells are larger than most cells. Upon osmotic or temperature shock these cells expel the lipid droplet and become indistinguishable from other large cells.

These cells were invariably large and round. Cells were plated on Greiner 6-well plates (Advanced TC surface polymer). Cell adhesion to plastic polymers has been widely investigated. Adhesion of cells to polysterene surfaces has been shown to be mediated by amphiliphilic peptides. Tissue culture plastic has a net negative surface charge which is produced by plasma treatment of the polystyrene. Adhering cells did not appear to possess more lipid droplets. Adhering cells sunk quite rapidly to the bottom of the dish. Non adhering cells took longer to sink, indicating a possible difference in buoyancy. Release of lipid droplets happened over time. No specific treatment appeared to affect the release process. As noted in Chapter 2, presence and release of lipid droplets had a considerable effect upon attempts to FACs sort cells. The lipid droplets appeared to be considerably responsible for the background observed. Macromeres appeared to be larger than any other cell present in the larva. All cells were spherical. Few exceptions to this rule were observed. Cells observed to demonstrate an elongated or non-spherical morphology appeared overall different, containing less ”clear cytoplasm” and taking up unusual shapes (Fig. 2.12). Overall, practically all cells were spherical, few cell stood out in terms of size difference. Due to high rate of cell division, cell identity correlated to size was deemed impossible to achieve.

Morphology of cells derived from post-12hpf larvae

Cells derived from 12hpf and 17 hpf larvae do not display any significant differences in terms of morphology. The only difference between cells derived from pre-10hpf larvae and 12-17hpf larvae is the presence of ciliated prototroch cells. These cells ”swim” in liquid media and sea water. They are the first cells to differentiate into differentiated cell types with easily recognizable morphological features and form new structures. Two tiers of twelve cells form at 10-12hpf at the equator of the trochophore larva. The presence of multiciliated cells in the larvae of organisms is widespread in the animal kingdom and is the defining characteristic of the Lophotrochozoan group. Cells have been described in some detail. In *Platynereis dumerilii*, prototroch cells have been investigated by Gaspar Jekely [308]. Two cells located at opposing lateral poles of the larval have been identified as ”steering” cells. These cells are directly innervated by neuronal projections from the larval eye, allow the swimming response of the larvae to be phototactic. Jekely and colleagues have demonstrated that ablation of the steering cells results in down-regulation of the phototaxis. The selective illumination of one

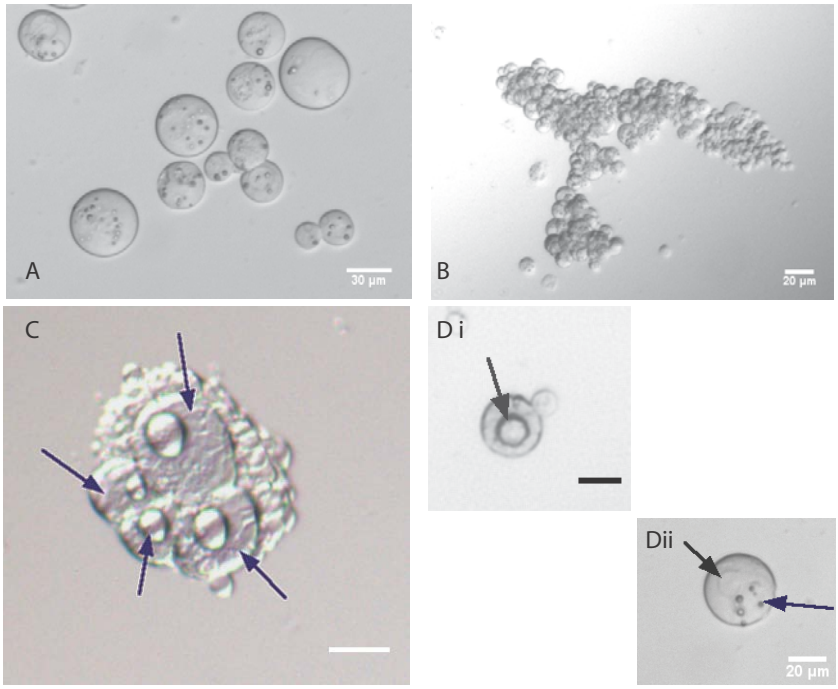


Figure 3.8: Cells derived from 10hpf-old larvae.A) Large cells in CMF seawater do not exhibit an specific morphology (B) Cells aggregate easily in SFNSW (C) Dechorionated young larva displaying four macromeres containing lipid droplets (Scale bar = 20μm) (Di-ii) Cells contain a distinguishable nucleus (Black arrow) and dark lipidic and granular structures (Blue arrow). (Dissociated macromere containing a single lipid droplet

larval "eyespot changes the beating of adjacent cilia by direct cholinergic innervation resulting in locally reduced water flow", thus regulating the phototaxis of the larva. A variety of morphologies of cells possessing cilia were observed (Fig. 3.11).

To test whether prototroch cells are phototactic themselves, dissociated cells were placed in the same chamber as the one used by Jekely and colleagues. Cell movement was analyzed using the ImageJ software (Fig. 3.12. Statistically significant phototactic-like or photophobic-like movement was not observed. Overall, 2/10 cells appeared to swim towards the light source, whereas the remaining cells invariably continued to swim in a manner unrelated to the light source.

Another interesting feature of these cells, was demonstrated independently by Dondua and Fischer that embryonic transcription of maternal transcripts is not necessary for the formation of prototroch, using actinomycin D treatment[309, 310]. Actinomycin D treatment was carried out as described by Dondua and Fischer. Indeed, although larvae appeared overall delayed in development and misdevelopped (absence of eye spots, overall smaller size, lack of swimming behavior), beating ciliary cells were observed (Fig. 3.13).

A further aspect of interest was the observation that if larvae were dissociated at stages prior to ciliogenesis (8 or 9 hpf), ciliated cells could be observed several hours later. This indicates that ciliogenesis does not require direct cell-to-cell communication, and that cell fate is determined up to several hours prior to the appearance of cilia. To determine whether any factors affect ciliary beating, whole larvae et 24-36 hours were incubated in several substances including: Putrescein HCl (NMDA agonist), Insulin, ITS, Taurin, α Tocopherol acetate (vitamin E), hydrocortisone and EGF. Curiously the only substance observed to stop ciliary beating was α adrenergic agonist Berberil (R), commercially available in the form of eye drops. Cilia ceased beating and became "rigid" (Fig. 3.14), uncoupling the ciliary beating of the trochophore cells within a single larva. The alpha adrenergic receptor is a G protein-coupled receptor (GPCR). This data is in line with data from vertebrates indicating that alpha-adrenergic agonists inhibit ciliary activity. [311].

For the most part, cells derived from post 15 hpf larvae displayed circular morphologies. With the exception of ciliated cells (Fig. 3.9 and Fig. 3.11), few cells displayed considerable morphological differences. Some cells displayed a slightly conical shape, and in some cases cellular projections that did not correspond to ciliated cells were observed (Fig. 3.9). Whether these cells corresponded to a precise cell type was not established (see Pilot RNA screen below).

Ciliary prototroch cells were observed in several sizes and shapes. As noted above, the prototroch cells in the larva display a parallelipipedic shape. None of the dissociated cells, however maintained this morphology. Cells displayed primarily a round morphology. The cilia could be distinctly observed in many cases. Cells derived from young larvae (24hpf) which are known to possess twelve prototroch cells, were slightly elongated in shape. Cells derived from older larvae (36-48hpf) included smaller cells which could correspond to either the metatroch cells which appear at later stages in development or the apical organ ciliated cell. As noted previously, complete dissociation was frequently not achieved and numerous cells remained firmly attached to another cell. This adhesion was strong enough to allow the 'passenger' cell to be transported along with the ciliated cell throughout its movement.

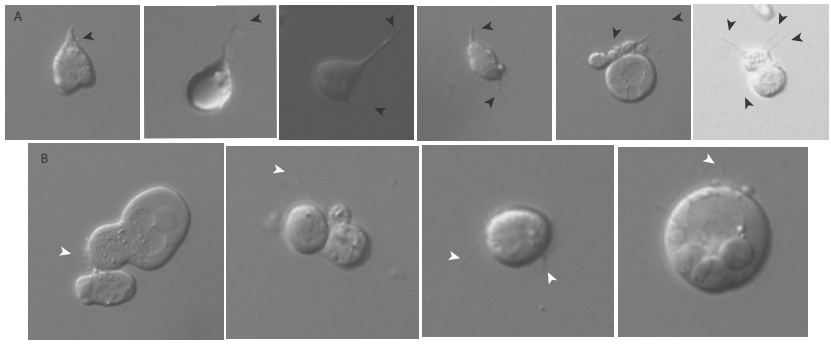


Figure 3.9: Cells displaying non-circular morphology from 24hpf larvae: (A) Cells with non-circular morphology with one or two projections. (B) Different morphologies displayed by ciliated cells.

During a preliminary observation of ciliated cells, three types of behavior were observed: (1) Circular swimming (Fig. 3.15A) (2) Straight-like movement(Fig. 3.15B) (3) Twirling(Fig. 3.15C)

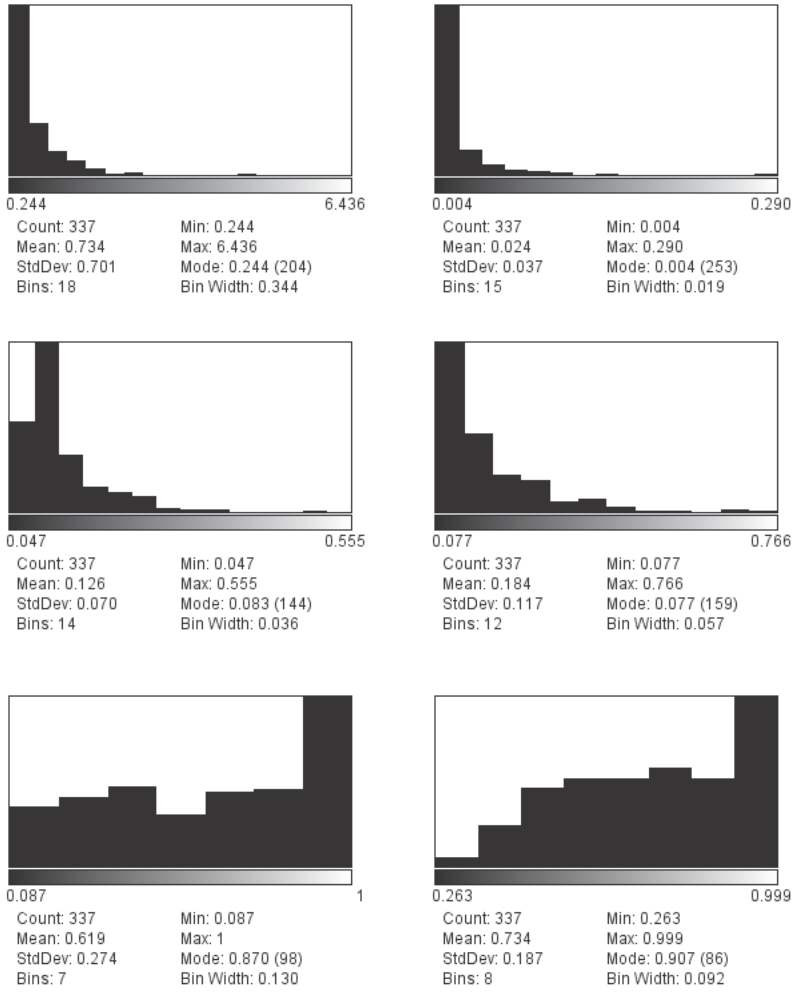


Figure 3.10: (A) Perimeter distribution (B)Area distribution (C) Minor axis length distribution (D) Major axis length distribution (E) Circularity distribution (F) Round distribution from a pool of 337 cells derived from larvae 12-15hpf-old) as determined using ImageJ software

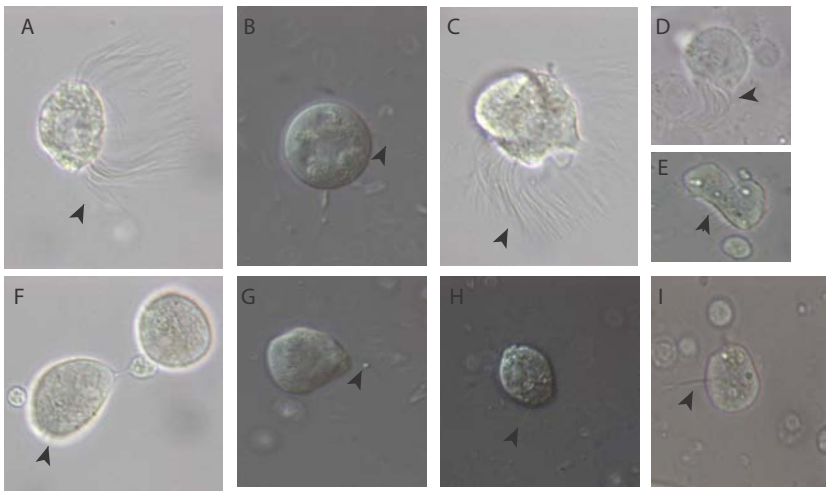


Figure 3.11: Variety of morphologies of ciliated cells from 48 hpf old trochophore larvae: (A) Undamaged but elongated prototroch cell with large nucleus (cilia indicated by black arrows) (B) Circular ciliated cell with granular contents (C) Ciliated prototroch cell from incomplete dissociation with attached non-ciliated cell (D) Damaged ciliated prototroch cell lacking cytoplasmic granules (E) Elongated ciliated cell possibly from apical tuft (F & H) Ciliated prototroch cell forming a cytoplasmic bridge to neighboring cell (G) Ciliated prototroch cell in the process of changing shape (H)Partially elongated ciliated prototroch cell

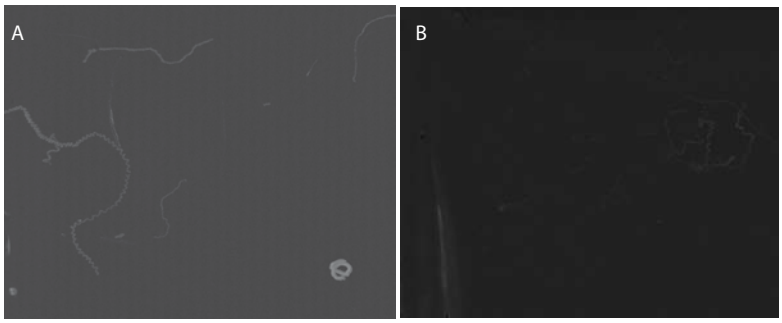


Figure 3.12: Cell phototaxis assay performed and recorded as described by [308] Light source was at the left. Figure shows maximal projections of timelapse recordings produced with ImageJ. (A and B) show two independent assays indicating no significant phototaxis. In the first assay (A) one cell was observed to approach light source (on left), whereas in a second assay (B) none were observed to approach or distance themselves from light source.

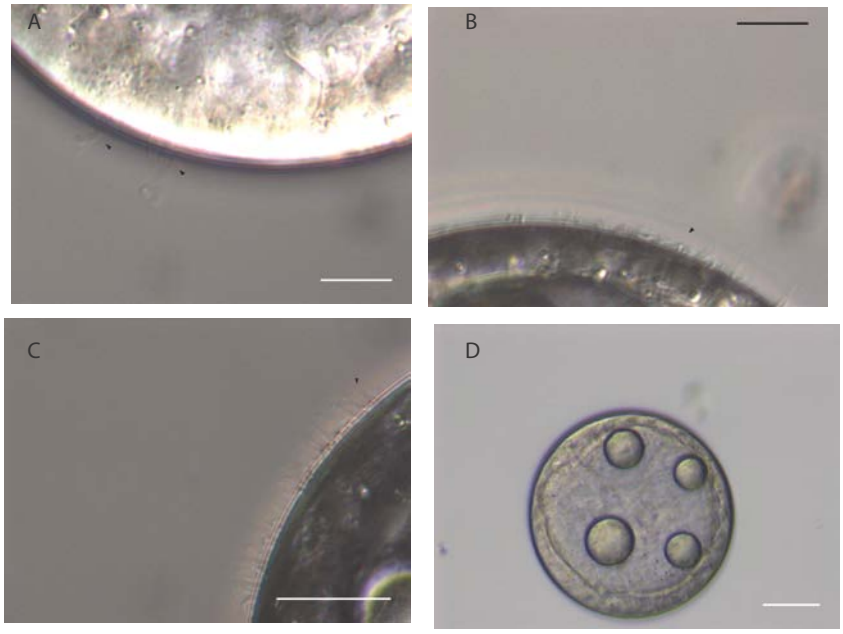


Figure 3.13: Effect of Actinomycin D on ciliated cell formation. Cilia formation was observed in 17hpf larvae. Cilia were observed to be present at full size (A) or slightly shorter (B) and ciliary beating was synchronous and functional in all larvae examined (C). General morphology appeared normal and four characteristic lipid droplets visibe(D).

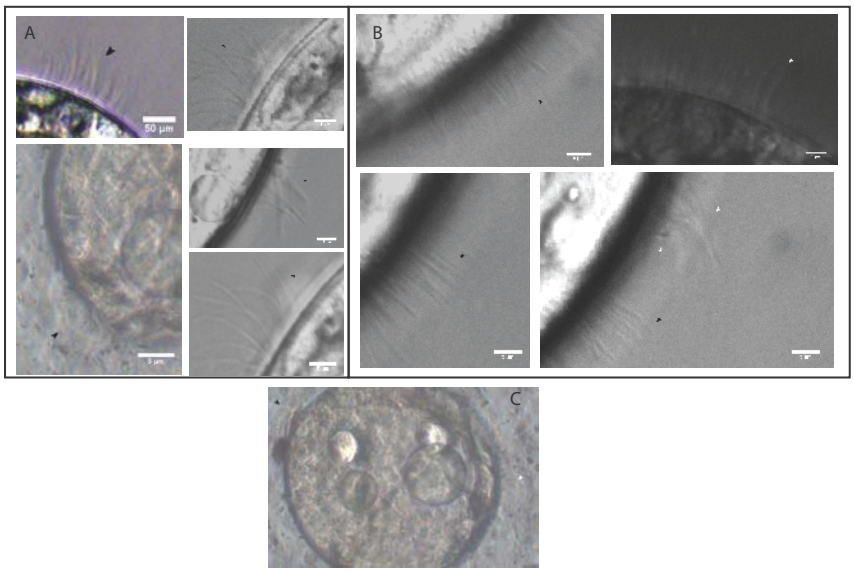


Figure 3.14: Effect of Berberil on ciliated cell beating. (Black arrow indicate normally beating cilia whereas white arrows indicate uncoupled cilia) (A) Normal ciliary beating was observed in non-treated larvae, whereas larvae incubated in two or more drops of Berberil were observed to stop ciliary beating in some cilia, whilst others continued beating (B)General morphology of larvae was not affected after 30 minutes incubation in the presence of Berberil.(C)

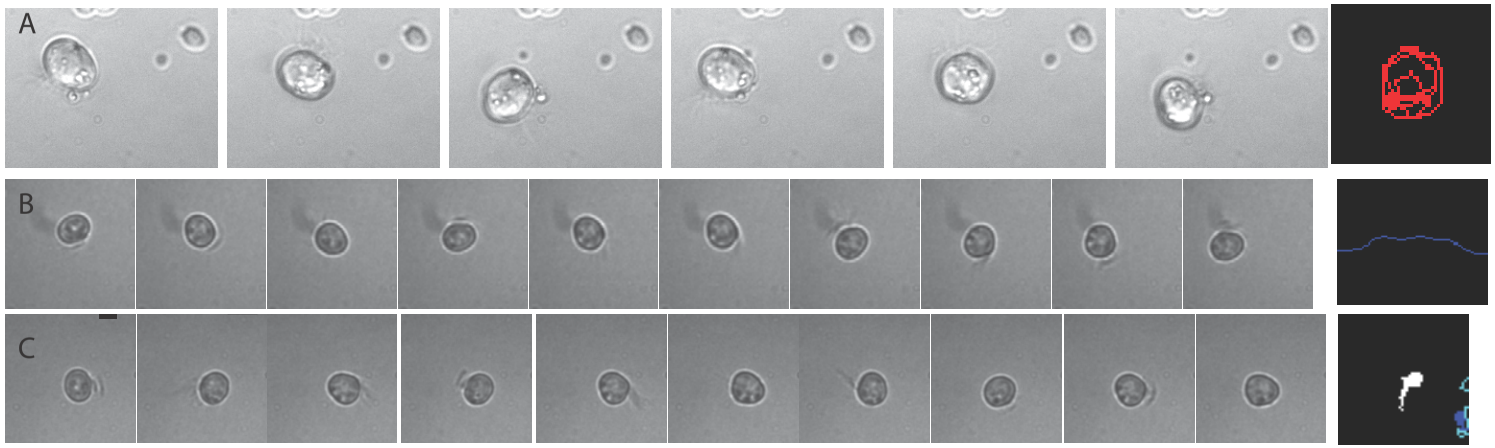


Figure 3.15: The three different swimming behaviors displayed by ciliated prototroch cells of *Platynereis dumerilii* recorded on high-speed camera. Sequences acquired every 20ms to left. To right, tracking of cell movement with ImageJ software over entire sequence. Imaging of cell (A) Cell swimming in a circular manner. (B) Cell swimming in a straight-like manner. (C) Cell "twirling" in one place.

One hypothesis could be a heterogenous distribution of cilia over the surface of the ciliated cell, in such a way that a force is being predominantly exerted on one side of the cell rather than another, resulting in a net movement in either a straight line when only force is dominant of movement in a straight line and in a circular movement when two opposing forces are applied on the cell. Observation of prototroch cells *in vivo* demonstrates a clear localization of all cilia on one side of the cell (the side in contact with the environment) and an absence of cilia on the faces of the cell in contact with the other cells of the larva. Upon dissociation, however the physical restraint exerted on the cell by its formerly neighboring cells is removed and the cell takes up a circular shape, distributing its cilia over its surface. Based on acetylated tubulin stainings of whole larvae, it can be assumed that all cells of the prototroch display identical cilia numbers, and therefore that distribution of cilia in each dissociated cell should be identical as well. The dissociation procedure as described above consists of enzymatic treatment which can be harmful to the cilia. If the proteolytic treatment of identical cells was responsible for the difference in swimming behavior then the number of cells displaying it should be random and the behavior of certain cells should not be statistically significant. In order to verify this, cells obtained from large dissociation procedures were tracked manually using ImageJ. The Net displacement/Total displacement was calculated for each cell. If the cell was to travel in a purely straight trajectory Net/Total displacement would be expected to correspond to 1. Cells traveling in small steps in a small trajectory would have a small value <1, whereas cells covering larger surfaces but arriving at a final point close to the start position would yield a number >1. Cells sampled at the same frequency and at the same magnification could be compared together to yield a statistically meaningful result. As the distribution of linearity did not not follow a standard distribution, a non-parametric test (two sample Kolmogorov-Smirnoff test)was used to compare the linearity between cells. Kolmogorov -Smirnov Two-Sample uses two independent samples X1, ..., Xn and Y1, ..., Yn with continuous distributions F(x) and G(x), respectively in order to test H0 F(x) for all x against HA F(x) not equal G(x). D corresponds to the largest difference between the distributions of samples tested. The p-value indicates the trustability of the test. At low p-values the null hypothesis H0 (samples are equal) is rejected in favour of the alternative hypothesis HA (samples are not equal) in two samples of cells recorded simultaneously in the same conditions consisting of 23 cells (Table 3.2.1) and 31 cells (Table 3.2.1). Judging from these results 1/7 cells differs significantly from its counterparts indicating that there may be an inherent difference between prototroch cells. A further hypothesis explaining different ciliary beating could be local minute differences in calcium concentrations. This was tested by incubating ciliated cells in media containing higher calcium concentrations such as DMEM and comparing them to cells incubated in Schneider medium containing less calcium ions (see Chapter 4). Cells were not observed to be more likely to swim in a linear fashion in low calcium conditions. Thus, this hypothesis could be rejected. It would therefore appear that there are at least two distinct populations of ciliated cells in *Platynereis dumerilii* early larvae, exhibiting statistically significant differences in behavior. The reason behind this difference is not clear.

		Cell1	Cell2	Cell3	Cell4	Cell5	Cell6	Cell7	Cell8	Cell9	Cell10	Cell11	Cell12	Cell13	Cell14	Cell15	Cell16	Cell17	Cell18	Cell19	Cell20	Cell21	Cell22	Cell23	Cell24	Cell25	Cell26	Cell27	Cell28	Cell29	Cell30	Cell31
Cell1	*D*	x	0.50	0.93	0.59	0.42	0.74	0.83	0.59	0.23	0.43	0.67	0.42	0.20	0.38	0.16	0.25	0.60	0.37	0.13	0.70	0.18	0.38	0.91	0.58	0.30	0.38	0.79	1.00	0.40	0.34	0.30
	p-value		0.00	¡ 0.00	0.00	0.00	¡ 0.00	0.00	0.00	0.02	0.00	¡ 0.00	0.00	0.06	0.00	0.17	0.01	0.00	0.00	0.41	¡ 0.00	0.12	0.00	¡ 0.00	0.00	0.00	0.00	¡ 0.00	0.00	0.00	0.00	0.00
Cell2	*D*	x	x	0.68	0.19	0.16	0.41	0.53	0.16	0.70	0.32	0.35	0.11	0.44	0.34	0.45	0.34	0.30	0.26	0.48	0.40	0.48	0.26	0.65	0.16	0.34	0.37	0.45	0.81	0.23	0.26	0.21
	p-value			¡ 0.00	0.10	0.23	0.00	0.00	0.25	¡ 0.00	0.00	0.00	0.69	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.00	0.26	0.00	0.00	0.00	0.00	0.02	0.01	0.06
Cell3	*D*	x	x	x	0.75	0.82	0.44	0.34	0.76	0.98	0.96	0.49	0.79	0.97	0.91	0.99	0.95	0.54	0.88	0.99	0.48	0.99	0.91	0.26	0.76	0.88	0.95	0.52	0.47	0.89	0.90	0.84
	p-value				¡ 0.00	¡ 0.00	0.00	0.01	¡ 0.00	¡ 0.00	¡ 0.00	0.00	¡ 0.00	¡ 0.00	¡ 0.00	¡ 0.00	¡ 0.00	0.00	¡ 0.00	¡ 0.00	¡ 0.00	0.00	¡ 0.00	¡ 0.00	0.03	¡ 0.00	¡ 0.00	¡ 0.00	0.00	0.01	¡ 0.00	¡ 0.00	¡ 0.00
Cell4	*D*	x	x	x	x	0.25	0.40	0.54	0.12	0.78	0.42	0.32	0.20	0.60	0.49	0.60	0.52	0.27	0.42	0.64	0.34	0.65	0.36	0.71	0.08	0.48	0.47	0.36	0.87	0.36	0.38	0.34
	p-value					0.01	0.00	0.00	0.52	¡ 0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	¡ 0.00	0.00	0.00	0.95	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cell5	*D*	x	x	x	x	x	0.52	0.66	0.26	0.65	0.23	0.44	0.20	0.35	0.26	0.38	0.26	0.40	0.19	0.42	0.47	0.41	0.18	0.78	0.22	0.29	0.30	0.51	0.93	0.14	0.18	0.16
	p-value						0.00	0.00	0.00	¡ 0.00	0.02	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.12	¡ 0.00	0.02	0.00	0.00	0.00	0.00	0.31	0.12	0.17
Cell6	*D*	x	x	x	x	x	x	0.20	0.37	0.90	0.71	0.13	0.38	0.81	0.71	0.81	0.71	0.18	0.65	0.85	0.10	0.82	0.65	0.41	0.40	0.73	0.74	0.25	0.63	0.59	0.65	0.59
	p-value							0.24	0.00	¡ 0.00	¡ 0.00	0.41	0.00	¡ 0.00	¡ 0.00	¡ 0.00	¡ 0.00	0.12	¡ 0.00	¡ 0.00	0.77	¡ 0.00	¡ 0.00	0.00	0.00	¡ 0.00	¡ 0.00	0.01	0.00	0.00	¡ 0.00	0.00
Cell7	*D*	x	x	x	x	x	x	x	0.50	0.95	0.84	0.24	0.50	0.89	0.81	0.92	0.83	0.30	0.74	0.93	0.24	0.92	0.77	0.31	0.53	0.83	0.82	0.31	0.54	0.70	0.74	0.67
	p-value								0.00	¡ 0.00	¡ 0.00	0.10	0.00	¡ 0.00	0.00	¡ 0.00	¡ 0.00	0.02	0.00	¡ 0.00	0.12	¡ 0.00	0.00	0.04	0.00	0.00	¡ 0.00	0.01	0.00	0.00	0.00	0.00
Cell8	*D*	x	x	x	x	x	x	x	x	0.79	0.47	0.34	0.20	0.55	0.45	0.56	0.46	0.25	0.38	0.58	0.33	0.59	0.40	0.71	0.13	0.47	0.48	0.36	0.88	0.33	0.40	0.32
	p-value									¡ 0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.41	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cell9	*D*	x	x	x	x	x	x	x	x	x	0.65	0.86	0.62	0.38	0.57	0.36	0.45	0.81	0.59	0.24	0.86	0.36	0.57	0.95	0.79	0.52	0.59	0.92	1.00	0.60	0.44	0.49
	p-value										¡ 0.00	¡ 0.00	0.00	0.00	0.00	0.00	0.00	¡ 0.00	0.00	0.01	¡ 0.00	0.00	0.00	¡ 0.00	0.00	0.00	0.00	¡ 0.00	¡ 0.00	0.00	0.00	0.00
Cell10	*D*	x	x	x	x	x	x	x	x	x	x	0.62	0.36	0.30	0.15	0.32	0.21	0.60	0.14	0.42	0.66	0.36	0.10	0.92	0.40	0.16	0.18	0.74	1.00	0.18	0.41	0.19
	p-value											0.00	0.00	0.00	0.23	0.00	0.04	0.00	0.31	0.00	¡ 0.00	0.00	0.77	¡ 0.00	0.00	0.17	0.12	¡ 0.00	¡ 0.00	0.12	0.44	0.08
Cell11	*D*	x	x	x	x	x	x	x	x	x	x	x	0.32	0.74	0.65	0.75	0.66	0.11	0.54	0.77	0.10	0.76	0.55	0.48	0.33	0.67	0.67	0.18	0.69	0.54	0.55	0.51
	p-value												0.00	¡ 0.00	¡ 0.00	¡ 0.00	¡ 0.00	0.64	0.00	¡ 0.00	0.77	¡ 0.00	0.00	0.00	0.00	¡ 0.00	¡ 0.00	0.12	0.00	0.00	0.00	0.00
Cell12	*D*	x	x	x	x	x	x	x	x	x	x	x	x	0.46	0.38	0.48	0.38	0.27	0.27	0.49	0.35	0.49	0.30	0.73	0.20	0.40	0.41	0.41	0.90	0.26	0.29	0.24
	p-value													0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.01
Cell13	*D*	x	x	x	x	x	x	x	x	x	x	x	x	x	0.23	0.10	0.18	0.69	0.30	0.19	0.79	0.09	0.30	0.95	0.56	0.23	0.27	0.84	1.00	0.32	0.44	0.29
	p-value														0.02	0.77	0.12	¡ 0.00	0.00	0.08	¡ 0.00	0.88	0.00	¡ 0.00	0.00	0.02	0.00	¡ 0.00	¡ 0.00	0.00	0.00	0.00
Cell14	*D*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	0.27	0.20	0.58	0.14	0.37	0.69	0.27	0.15	0.89	0.45	0.10	0.09	0.76	0.98	0.18	0.44	0.16
	p-value															0.00	0.06	0.00	0.31	0.00	¡ 0.00	0.00	0.23	¡ 0.00	0.00	0.77	0.88	¡ 0.00	0.00	0.12	0.77	0.17
Cell15	*D*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	0.18	0.69	0.32	0.15	0.79	0.12	0.30	0.95	0.55	0.25	0.30	0.87	1.00	0.35	0.25	0.30
	p-value																0.12	¡ 0.00	0.00	0.23	¡ 0.00	0.52	0.00	¡ 0.00	0.00	0.01	0.00	¡ 0.00	¡ 0.00	0.00	0.21	0.00
Cell16	*D*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	0.59	0.16	0.23	0.69	0.22	0.18	0.93	0.47	0.13	0.19	0.76	1.00	0.22	0.25	0.20
	p-value																	0.00	0.17	0.02	¡ 0.00	0.02	0.12	¡ 0.00	0.00	0.41	0.08	¡ 0.00	¡ 0.00	0.02	0.33	0.06
Cell17	*D*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	0.52	0.73	0.18	0.71	0.53	0.51	0.29	0.59	0.60	0.23	0.70	0.47	0.42	0.45
	p-value																		0.00	¡ 0.00	0.12	¡ 0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00
Cell18	*D*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	0.36	0.60	0.33	0.09	0.85	0.38	0.16	0.18	0.67	0.93	0.12	0.10	0.12
	p-value																			0.00	0.00	0.00	0.87	¡ 0.00	0.00	0.17	0.12	¡ 0.00	0.00	0.52	0.71	0.52
Cell19	*D*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	0.82	0.21	0.35	0.95	0.60	0.29	0.36	0.87	1.00	0.38	0.53	0.32

	p-value																					¡ 0.00	0.04	0.00	¡ 0.00	0.00	0.00	0.00	¡ 0.00	0.00	0.00	0.00	0.00
Cell20	*D*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	0.80	0.60	0.44	0.33	0.70	0.69	0.16	0.64	0.58	0.62	0.55
	p-value																					¡ 0.00	0.00	0.00	0.00	¡ 0.00	¡ 0.00	0.17	0.00	0.00	0.00	0.00
Cell21	*D*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	0.34	0.94	0.60	0.25	0.29	0.87	0.99	0.34	0.29	0.33
	p-value																						0.00	¡ 0.00	0.00	0.01	0.00	¡ 0.00	0.00	0.00	0.00	0.00
Cell22	*D*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	0.88	0.34	0.18	0.19	0.67	0.99	0.11	0.09	0.13
	p-value																							¡ 0.00	0.00	0.12	0.08	¡ 0.00	0.00	0.64	0.37	0.41
Cell23	*D*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	0.72	0.87	0.90	0.57	0.24	0.85	0.86	0.79
	p-value																								0.00	¡ 0.00	¡ 0.00	0.00	0.51	¡ 0.00	¡ 0.00	0.00
Cell24	*D*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	0.45	0.44	0.37	0.88	0.34	0.35	0.31
	p-value																									0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cell25	*D*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	0.11	0.78	0.95	0.20	0.16	0.18
	p-value																										0.64	¡ 0.00	0.00	0.06	0.17	0.12
Cell26	*D*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	0.79	1.00	0.19	0.18	0.18
	p-value																											¡ 0.00	¡ 0.00	0.08	0.12	0.12
Cell27	*D*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	0.76	0.64	0.67	0.64
	p-value																												0.00	0.00	¡ 0.00	0.00
Cell28	*D*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	0.99	0.99	0.92
	p-value																													0.00	0.00	0.00
Cell29	*D*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	0.13	0.18
	p-value																														0.41	0.12
Cell30	*D*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	0.12
	p-value																															0.52
Cell31	*D*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	p-value																															

Table 3.1: Comparison of swimming behavior of ciliated cells evaluated by the Kolmogorov-Smirnov test

		Cell1	Cell2	Cell3	Cell4	Cell5	Cell6	Cell7	Cell8	Cell9	Cell10	Cell11	Cell12	Cell13	Cell14	Cell15	Cell16	Cell17	Cell18	Cell19	Cell20	Cell21	Cell22	Cell23
Cell1	*D*	X	0.77	0.93	0.21	0.50	0.36	0.50	0.25	0.21	0.29	0.21	0.29	0.50	0.57	0.86	0.71	0.50	0.57	0.43	0.36	0.29	0.43	0.79
	p-value		0.00	0.00	0.90	0.06	0.36	0.06	0.91	0.90	0.62	0.90	0.62	0.06	0.02	0.00	0.00	0.06	0.02	0.15	0.33	0.62	0.15	0.00
Cell2	*D*	X	X	0.26	0.79	0.43	0.92	0.43	0.63	0.71	0.93	0.79	0.79	1.00	0.47	0.24	1.00	0.40	1.00	0.86	0.42	0.63	1.00	1.00
	p-value			0.65	0.00	0.10	0.00	0.10	0.02	0.00	0.00	0.00	0.00	0.00	0.08	0.76	0.00	0.18	0.00	0.00	0.13	0.01	0.00	0.00
Cell3	*D*	X	X	X	0.79	0.50	0.93	0.50	0.68	0.86	0.86	0.79	0.86	0.93	0.50	0.29	1.00	0.43	1.00	0.86	0.57	0.79	0.93	1.00
	p-value				0.00	0.06	0.00	0.06	0.01	0.00	0.00	0.00	0.00	0.00	0.06	0.64	0.00	0.15	0.00	0.00	0.02	0.00	0.00	0.00
Cell4	*D*	X	X	X	X	0.43	0.32	0.43	0.29	0.14	0.43	0.36	0.36	0.71	0.43	0.79	0.79	0.57	0.71	0.50	0.50	0.29	0.57	0.79
	p-value					0.15	0.40	0.15	0.72	1.00	0.15	0.34	0.34	0.00	0.15	0.00	0.00	0.02	0.00	0.06	0.06	0.62	0.02	0.00
Cell5	*D*	X	X	X	X	X	0.57	0.21	0.34	0.50	0.57	0.43	0.43	0.71	0.21	0.43	0.86	0.21	0.71	0.57	0.14	0.36	0.71	0.86
	p-value						0.01	0.92	0.50	0.06	0.02	0.15	0.15	0.00	0.92	0.15	0.00	0.92	0.00	0.02	1.00	0.33	0.00	0.00
Cell6	*D*	X	X	X	X	X	X	0.57	0.38	0.29	0.26	0.21	0.27	0.47	0.70	0.93	0.62	0.71	0.48	0.34	0.64	0.43	0.32	0.71
	p-value							0.01	0.39	0.64	0.74	0.81	0.59	0.08	0.00	0.00	0.01	0.00	0.07	0.34	0.00	0.17	0.42	0.00
Cell7	*D*	X	X	X	X	X	X	X	0.32	0.43	0.50	0.36	0.43	0.64	0.36	0.43	0.79	0.29	0.64	0.43	0.21	0.36	0.57	0.79
	p-value								0.55	0.15	0.06	0.34	0.15	0.00	0.34	0.15	0.00	0.64	0.00	0.15	0.92	0.33	0.02	0.00
Cell8	*D*	X	X	X	X	X	X	X	X	0.25	0.36	0.21	0.18	0.43	0.43	0.68	0.61	0.50	0.50	0.36	0.36	0.29	0.43	0.66
	p-value									0.91	0.53	0.94	0.98	0.24	0.24	0.01	0.03	0.12	0.12	0.46	0.46	0.80	0.24	0.02
Cell9	*D*	X	X	X	X	X	X	X	X	X	0.36	0.29	0.21	0.64	0.50	0.86	0.71	0.50	0.64	0.50	0.36	0.29	0.57	0.64
	p-value										0.33	0.62	0.90	0.01	0.06	0.00	0.00	0.06	0.01	0.06	0.33	0.62	0.02	0.01
Cell10	*D*	X	X	X	X	X	X	X	X	X	X	0.21	0.21	0.29	0.64	0.93	0.64	0.71	0.36	0.29	0.57	0.50	0.29	0.57
	p-value											0.90	0.90	0.62	0.01	0.00	0.01	0.00	0.33	0.62	0.02	0.06	0.62	0.02
Cell11	*D*	X	X	X	X	X	X	X	X	X	X	X	0.21	0.43	0.64	0.79	0.57	0.50	0.43	0.29	0.43	0.36	0.29	0.64
	p-value												0.92	0.15	0.00	0.00	0.02	0.06	0.15	0.64	0.15	0.33	0.64	0.01
Cell12	*D*	X	X	X	X	X	X	X	X	X	X	X	X	0.43	0.43	0.79	0.50	0.57	0.43	0.29	0.43	0.29		0.57
	p-value													0.15	0.15	0.00	0.06	0.02	0.15	0.62	0.15	0.62		0.02
Cell13	*D*	X	X	X	X	X	X	X	X	X	X	X	X	X	0.86	1.00	0.43	0.79	0.50	0.36	0.71	0.57		0.43
	p-value														0.00	0.00	0.15	0.00	0.06	0.34	0.00	0.02		0.15
Cell14	*D*	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0.43	0.93	0.14	0.86	0.71	0.21	0.36	0.86	0.93
	p-value															0.15	0.00	1.00	0.00	0.00	0.92	0.33	0.00	0.00
Cell15	*D*	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	1.00	0.43	1.00	0.86	0.50	0.71	1.00	1.00
	p-value																0.00	0.15	0.00	0.00	0.06	0.00	0.00	0.00
Cell16	*D*	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0.93	0.64	0.57	0.86	0.71	0.36	0.43
	p-value																	0.00	0.00	0.02	0.00	0.00	0.34	0.15
Cell17	*D*	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0.79	0.64	0.21	0.36	0.79	0.93
	p-value																		0.00	0.00	0.92	0.33	0.00	0.00
Cell18	*D*	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0.29	0.71	0.64	0.43	0.79
	p-value																			0.64	0.00	0.01	0.15	0.00
Cell19	*D*	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0.57	0.50	0.36	0.64
	p-value																				0.02	0.06	0.34	0.01
Cell20	*D*	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0.21	0.64	0.86
	p-value																					0.90	0.00	0.00
Cell21	*D*	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0.57	0.71
	p-value																						0.02	0.00
Cell22	*D*	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0.50
	p-value																							0.06
Cell23	*D*	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	p-value																							

Table 3.2: Comparison of swimming behavior of ciliated cells evaluated by the Kolmogorov-Smirnov test

Substance used	What it stains	Success
DAPI	Nucleus	-
Hoechst	Nucleus	+
DRAQ5	Nucleus	+
SYBR	Nucleic acid	+
Mitotracker	Mitochondria	-
Phalloidin	Actin	-
Acridine Orange	pH based staining	+

Table 3.3: Substances used to stain living *Platynereis dumerilii ex vivo*

3.2.2 Molecular characterization of *Platynereis dumerilii*-derived cells

Development of immunohistochemical characterization of living *Platynereis dumerilii*-derived cells

Platynereis dumerilii larvae and adults have been extensively characterized using WMISH and immunohistochemistry, at up to a single cell resolution [31]. However, few descriptions are available of *Platynereis* cell characterization *ex vivo*. In order to characterize living cells, several substances were tested, with varying success (Table 3.2.2, Fig. 3.16.

Development of immunohistochemical characterization of fixed *Platynereis dumerilii*-derived cells

Cell adhesion Several protocols were assayed for the staining of *Platynereis* cells. The first problem consisted in the necessity to firmly attach cells to a substrate in order to avoid cell loss during successive washes. For this reason, the methods tested in Chapter 4 (Adhesion assay) were tested. The best results were observed a method developed by M. Schroer [312]. Two plexiglass plaques approximately the same size as a 96 well plate, fixed together using screws, one containing 9mm-deep holes at the bottom of which coverslips could be place. The cell suspension could be added into the holes in the plexiglass. After centrifugation at 1000x rpm cells firmly attached to the coverslips at bottom of the wells formed by the holes in the plexiglass. Moreover, cell adhesion assays were performed using different substrates (see Chapter 4).

After cells could be confidently attached to the surface of coverslips, efforts were invested into testing fixation and permeabilization methods.

Prioir to fixing cells, coverslips were placed in either Greiner 96-well plates or 24-well plates. Alternatively attempts were made to perform staining using 100 or 200 μl drops distributed on sheet of parafilm surrounding a plexiglass block, by transferring coverslips from one drop to another. Although all steps lasting over 20 minutes were performed in a sealed box containing humidified paper towel, attempts to stain cells using the drop method invariably worked less well than when the coverslips were entirely covered by solution.

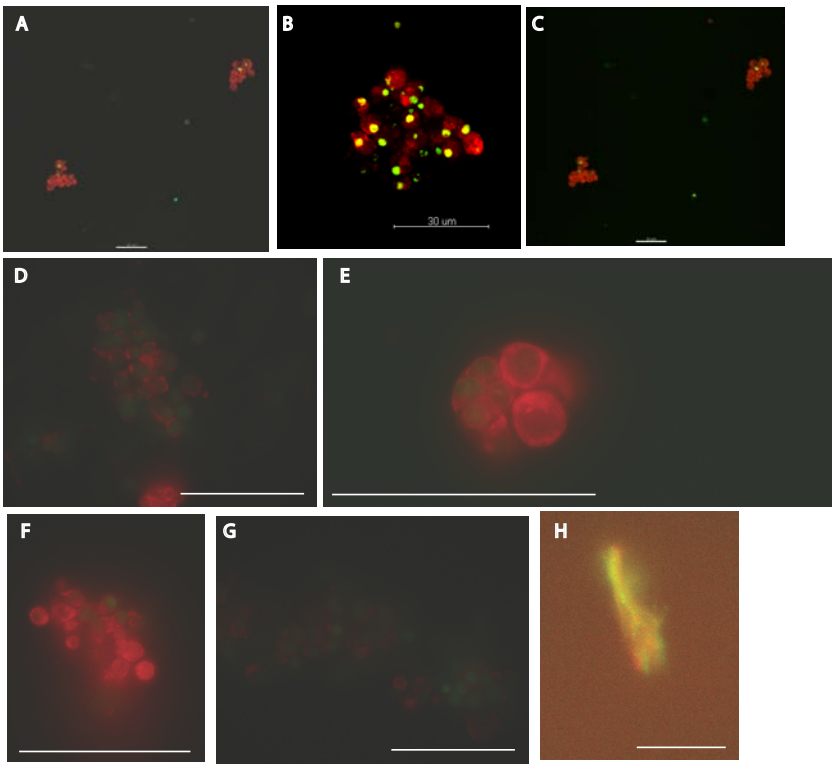


Figure 3.16: (Acridine orange staining on living *Platynereis* cells (A-C) *Platynereis* larval cell aggregates stained with acridine orange visualized with an SPE confocal microscope and rendered using ImageJ (D-K) *Platynereis* adult cells stained with acridine orange visualized with a fluorescent microscope (K) *Platynereis* adult muscle cell stained with acridine orange

Fixation Fixation was attempted using several solutions. The standard solution utilized in *Platynereis* larval immunocytochemistry [313] consists of a 4% PFA in PBS Tween solution. Solution is prepared using 3 volumes of 2x PTW stock solution (0.1% Tween 20) and 16% paraformaldehyde. The protocol utilized on wholemount larvae immunocytochemistry (without the proteinase K digestion step) did not yield acceptable results. Permeabilization of cells 8%, 2% and 1% PFA and formaldehyde were tested in PBS and using 0.5%, 1%, 2%, 4% and 5% Tween 20, Triton, saponin (curtesy of Dr. Ghislaine Arib from the Akhtar lab, EMBL Heidelberg, MPI Freiburg) and Nonidet NP-40were tested. Wash steps were performed for 3x 5 minutes, 5x 5 minutes between solutions or overnight after antibody wash steps. Fixation was attempted for 5, 10, 15, 20, 30 and 45 minutes. Fixation efficiency was evaluated based on conservation of cell morphology and efficiency at producing a specific staining. Saponin and Nonidet NP-40 did not appear to confer increased specificity to staining during fixation. However, if all subsequent wash steps were performed in a solution of PBS containing the same detergent, increased permeabilization was indeed observed. Fixation for less than 15 minutes was judged insufficient. Cells disattached from the coverslips during subsequent washes resulting acceptable staining but in few cells that remained adherent to observe. Fixation for over 40 minutes did not yield acceptable staining in all cases tested. The best fixation conditions appeared to be 2%, 4% PFA and formaldehyde, in 2% Triton or Tween 20.

Washes However, staining with washing steps containing Triton were unsuccessful, independently of the durations of the wash steps tested. Overnight wash steps were unsuccessful. 5 x 5 minute wash steps resulted in excessive cell loss. Therefore, although non-specific staining was encountered more frequently using 3 x 5 minute wash steps, this protocol was judged better as a higher yield of cells was available. The highest impact in terms of conditions, was the duration of antibody incubation. Antibodies were utilized at 1:200. Incubation for 1 hour at room temperature, 2 hours at room temperature, 2 hours at $37\,^{\circ}\mathrm{C}$ and overnight at $4\,^{\circ}\mathrm{C}$.

Methanol Methanol permeabilization was attempted both stepwise (with 5 minute 25%:75% , 50%:50% and 75%:25% methanol:PBS Tween solution)and by placing fixed cells directly into 100% methanol solution. Methanol permeabilization was attempted for 20 minutes at $-20\,^{\circ}\mathrm{C}$ and overnight. In both cases, methanol treatment did not yield successful staining. Background signal was indistinguishable form specific signal.

Protocol The protocol that was judged most appropriate is similar to that described by Odintsova, Plotnikov and Karpenko [133].

A small-scale screen using a set of antibodies available in the lab was performed to determine whether antibodies were functional in cell culture with the above-mentioned protocol and to determine whether cells expressing any of a set of antigens of interest to the lab could be successfully recovered after cell dissociation. The results were disappointing. Stainings could be obtained with phalloidin and acetylated tubulin from

Fixation:	2%, 4% PFA and formaldehyde, in 1% or 2% Triton or Tween 20 for over 15 minutes but under 40 minutes.
Wash:	3 x 5 minutes PBS with in 1% or 2% Triton or Tween 20
Block:	depending on antibody 5% sheep or goat serum in PBS with in 1% or 2% Triton or Tween 20
Primary antibody incubation:	O/N incubation at $4\,^{\circ}\mathrm{C}$ in block solution at a dilution of 1:200
Wash:	3 x 5 minutes PBS with in 1% or 2% Triton or Tween 20
Secondary antibody:	O/N incubation at $4\,^{\circ}\mathrm{C}$ in block solution at a dilution of 1:200; No block step
Wash:	3 x 5 minutes PBS with in 1% or 2% Triton or Tween 20
Postfix:	10 minutes in 2%, 4% PFA and formaldehyde, in 1% or 2% Triton or Tween 20

cells from all stages of larvae tested (12hpf, 24-30 hpf, 50 hpf and 72 hpf). Serotonin stainings were rare and high background was observed. Curiously, acetylated tubulin stainings demonstrated a highly interconnected network of cellular protrusions between cells(Fig. 3.17). It is curious to compare these tubulin-based protrusions with the putative pseudopodia observed in dissociated blastomeres (Fig. 2.12). These large networks of projections containing tubulin were only observed in large cell aggregates containing over 8 or 10 clearly distinguishable nuclei. Small cell clusters containing 2-3 cells lacked these networks. The presence of such cellular connections may explain the difficulty in dissociating these cell clusters and in their growth in clusters.

Antibody or Stain Name	Company	Dilutions tested	Result
Mouse anti-GABA	AFFINITI	1:100, 1:200, 1:1000	-
Rabbit anti-γ-interferon	N/A	1:100, 1:200, 1:1000	-
Rabbit anti-histamine	N/A	1:100, 1:200, 1:1000	-
Rabbit antii-Phospho-c-jun (ser 73)-R	Cruz Biotechnology	1:100, 1:200, 1:1000	-
Anti-human CD57/HNK-1 Clone VC1.1	Sigma	1:100, 1:200, 1:1000	-
Rabbit Anti-luteinizing hormone releasing hormone	N/A	1:100, 1:200, 1:1000	-
Anti-Gonadotropin releasing Hormone(539)	Sigma	1:100, 1:200, 1:1000	-
Anti-Thyroxin [XM212]	ABcam	1:100, 1:200, 1:1000	-
Anti-NOS	N/A	1:100, 1:200, 1:1000	-
Phalloidin 488	Molecular Probes	1:100, 1:200, 1:1000	1:100, 1:200 +++
Phalloidin Rhodamine	Molecular Probes	1:100, 1:200, 1:1000	1:100, 1:200 +++
5HT Rabbit Serotonin	Abcam	1:100, 1:200, 1:1000	faint signal from cells in culture less than 12h from 48+hpf larvae
Rat Anti-tropomyosin	N/A	1:100, 1:200, 1:1000	-
Anti- GAD	N/A	1:100, 1:200, 1:1000	-
Anti-CamK	N/A	1:100, 1:200, 1:1000	-
Anti-Physin	N/A	1:100, 1:200, 1:1000	-
Mouse Anti- MAP2	Sigma	1:100, 1:200, 1:1000	-
Anti-acetylated tubulin	Sigma	1:100, 1:200, 1:1000	1:100, 1:200 very faint signal from post 40 hpf larvae
Rabbit anti-DjTH antibody	MDB Kyoto	1:100, 1:200, 1:1000	-
Mouse anti-DjTBH antibody	MDB Kyoto	1:100, 1:200, 1:1000	-
Mouse anti-DjGAD antibody	MDB Kyoto	1:100, 1:200, 1:1000	-
Mouse anti-DjTPH antibody	MDB Kyoto	1:100, 1:200, 1:1000	-
Mouse anti-DjChAT antibody	MDB Kyoto	1:100, 1:200, 1:1000	-
Rabbit anti-phospho-Histone H3 (S10)	ABcam	1:100, 1:500, 1:1000	1:500 +++
Rabbit Anti-β Catenin	Sigma	1:100, 1:200, 1:1000	very faint signal at under 24 hpf
Antiserum anti-PduC-5205 (rabbit 7538)	Biogenes GmbH	1:100, 1:250, 1:1000	-
Antiserum anti-Pdu-r- 5206 (rabbit 7541, 7540	Biogenes GmbH	1:100, 1:250, 1:1000	-
Antiserum Pdu-C-5204 (rabbit 7536, 7537)	Biogenes GmbH	1:100, 1:250, 1:1000	-
Antiserum c-opsin 7536 1st bleed	Biogenes GmbH	1:100, 1:250, 1:1000	-
Antiserum pdu c 5905 (rabbit 77538, 39) final bleed	Biogenes GmbH	1:100, 1:250, 1:1000	-
Antiserum anti-pdu 6498 (animal 9469, 9471) Serum	Biogenes GmbH	1:100, 1:250, 1:1000	-
Antiserum anti-6498 (9470)	Biogenes GmbH	1:100, 1:250, 1:1000	-
Antiserum anti-Pdu-c-5204 (rabbit 7537) final bleed	Biogenes GmbH	1:100, 1:250, 1:1000	-
Antiserum anti pdu-c-5204 (7537) final	Biogenes GmbH	1:100, 1:250, 1:1000	-
Total IgG fraction from anti5204	Biogenes GmbH	1:100, 1:250, 1:1000	-
Antiserum anti-pdu 6498 (animal 9469, 9470) CVFRI	Biogenes GmbH	1:100, 1:250, 1:1000	-
Antiserum 4032 spec. pur. 20 May anti-vglut	Biogenes GmbH	1:100, 1:250, 1:1000	-
Antiserum anti-vglut 04.09	Biogenes GmbH	1:100, 1:250, 1:1000	-
Antiserum 4032 serum Vglut	Biogenes GmbH	1:100, 1:250, 1:1000	-
Rabbit anti-PACA 27 NH2 (Human Ovin Rat)	Phoenix Pharmaceuticals 1:250	1:100, 1:250, 1:1000	-
Antiserum anti-somatostatin	Phoenix Pharmaceuticals 1:250	1:100, 1:250, 1:1000	-
Antiserum anti-FMRFamide	Phoenix Pharmaceuticals 1:250	1:100, 1:250, 1:1000	-
Antiserum anti-Pdu-N-Rx 4th	Phoenix Pharmaceuticals 1:250	1:100, 1:250, 1:1000	-
Antiserum Pdu-C-Rx 4th serum	Phoenix Pharmaceuticals 1:250	1:100, 1:250, 1:1000	-
Antiserum Pdu-c-Prox 4th serum	Phoenix Pharmaceuticals 1:250	1:100, 1:250, 1:1000	-
Serum 8B4D2 (MH2b)-s 1ea 5/10/07-31uh/ml Ig	Hybridoma bank	1:100, 1:250, 1:1000	-
Mabdac1-1-s 1ea 8/31/06-36ug/ml Ih	Hybridoma bank	1:100, 1:250, 1:1000	-
Anti-chicken Pax6-s 1ea 9/18/08-50ug/ml Ig	Hybridoma bank	1:100, 1:250, 1:1000	-

Anti-Syt-s 1ea 11/19/99-18ug/ml Ig Serum	Hybridoma bank	1:100, 1:250, 1:1000	-	
XAP-1 (Clone 3D2)-s 1ea 7/9/07-38ug/ml Ig	Hybridoma bank	1:100, 1:250, 1:1000	-	
ChAT4B1-s 1ea 9/18/07-24ug/ml	Hybridoma bank	1:100, 1:250, 1:1000	-	
4C5-s 1ea 3/1/07-18ug/ml	Hybridoma bank	1:100, 1:250, 1:1000	-	
GAD-6-s 1ea 10/2/07-32ug/ml Ig	Hybridoma bank	1:100, 1:250, 1:1000	-	
H5-s 1ea 3/5/08-34ug/ml Ig	Hybridoma bank	1:100, 1:250, 1:1000	-	
mMyC myomesin B4-s 1 ea 12/7/06-44ug/ml Ig	Hybridoma bank	1:100, 1:250, 1:1000	-	
D7F2-s 1ea 8/9/07-35ug/ml Ig Serum	Hybridoma bank	1:100, 1:250, 1:1000	-	
SUK-4-s 1ea 3/9/06-58ug/ml Ig Serum	Hybridoma bank	1:100, 1:250, 1:1000	-	
5F10 Serum Dipni-AST 7	Hybridoma bank	1:100, 1:250, 1:1000	-	
Synapsin Serum	Hybridoma bank	1:100, 1:250, 1:1000	-	
Myosin III	N/A	1:100, 1:250, 1:1000	-	
Mouse anti-diphospho SAPK/JNK (Thr185)	ABCam	1:100, 1:250, 1:1000	-	
Mouse anti-phospho Smad 1/5/8 (Ser 463/465)	ABCam	1:100, 1:250, 1:1000	-	

Table 3.4: List of antibodies used to perform immunocytochemistry screen on —Platyneteis cells and results of screen

Morphological characterization of *Platynereis* adult cells Cells obtained from adult *Platynereis* were much more numerous. These cells were often contaminated by large unicellular organisms. This can be explained by their original presence in the gut of the animal and subsequent liberation upon dissociation. Most of the cells observed from the coelomic fluid corresponded to elaeocytes. These cells appeared to be surrounded by large shiny viscous lipid-like substance. Attempts made to obtain *Platynereis* nerve cells were unsuccessful. Adult muscle cells were readily recognizable. Two populations of cells were observed those that "twitched" and those that "contracted". It was hypothesized that the first group corresponded to striated muscle whereas the second group corresponded to gut smooth muscle. Contraction speed could be quantified. *Platynereis* adult cells unlike larval cells demonstrated extreme variability in color and morphology.

RNA characterization study

During the development of dissociation methods, several QC steps were implemented to confirm that cells were indeed "alive" (Chapter 2). Viability tests were performed demonstrating that cell metabolism and active transport were active. Blastomeres derived from dissociated cells were observed to continue dividing, and therefore "alive". Ciliated prototroch cells were observed to continue "swimming". Moreover, staining with phosphotungstic acid and light microscopy observations (see Chapter 2), indicated that correct intracellular ooplasmic segregation took place during and after dissociation. In order to ascertain that mRNA could be retrieved from obtained from isolated *Platynereis* cells, a pilot study was carried out using a commercially available kit, the CellsDirect Superscript III (Invitrogen) kit. This kit is designed to produce single stranded cDNA from cultured cells. Manufacturer's employed methods similar to those described by Brady and Iscove, producing reverse transcribed cDNA complementary to the polyadenylated mRNA obtained from the lyzed cell(s). As ciliated cells of the prototroch are quite characteristic in their morphology and indications of several marker transcripts were available, these cells were selected for the test.

Development of QC primer set Accurate and rigorous quality control (QC) is of utmost importance when dealing with delicate experiments such as attempts to amplify single cell amounts of RNA. However, when these experiments were initiated no genomic resource was available for *Platynereis dumerilii*. As little information is available about gene expression in the cell types selected (based on morphologically easy recognition), confirmation of RNA quality was hampered. The *Platynereis* Expression Pattern Database produced by Dr. Kristin Tessmar-Raible, Dr. Thorsten Henrich, Dr. Mirana Ramialson and Mr.Andreas Scheucher, was used to find ESTs expressed in ciliated cells. Dr. Antje Fischer has studied gene expression of candidate transcription-factor encoding transcripts throughout development using an "absolute quantification" method. Based on her results candidate qPCR primers that had been shown in her hands to not result non-specific amplification and to provide signal with cDNA derived from oocytes.

Primers designed originally for reference "housekeeping" genes in *Platynereis* by Dr. Tomas Larsson and Dr. Heather Marlow. qRT-PCR using SYBR green allows

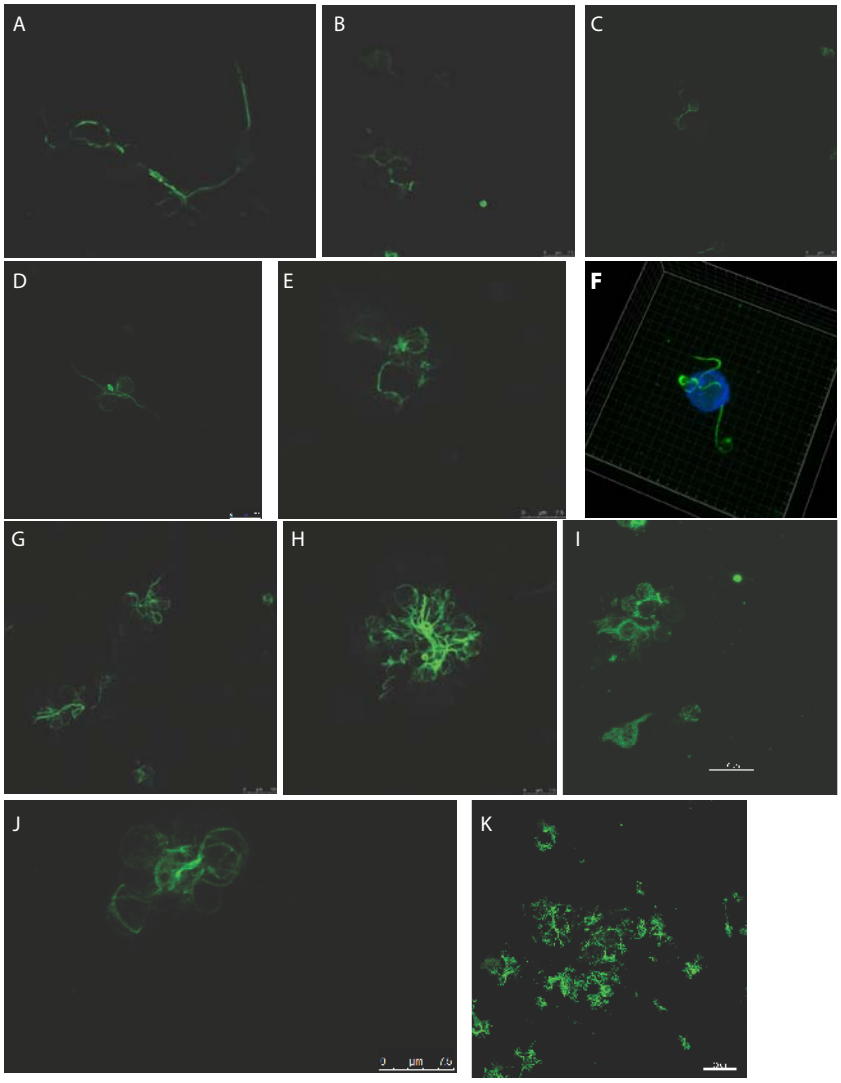


Figure 3.17: Examples of stainings with acetylated tubulin, demonstrating long tubulin-based cellular protrusions interconnecting cell aggregates (A-E) Tubulin-based processes connecting cells in different clumps (F) Tubulin (Green) and DAPI (blue) on a single cell with projections reaching out of the cell (G-K) Multiple cell aggregates interconnected by tubulin-containing projections

gene-specific amplification of minute quantities, however unlike the use of Taqman primers, primer-dimers may form. To ensure that observed signal corresponds to true amplification, qPCR is followed by a Melting curve. Primer dimer formation is often observed when primers are present in extreme excess of template. Therefore, primer dilution is necessary to amplify low abundance cDNA. This is performed by diluting each primer in presence and absence of template to determine whether unspecific product formation is observed. Optimal qPCR primer concentrations correspond to those where no non-specific amplification is observed(Table **??**. The primers used for this assay were originally designed as reference primers necessary for normalization. This purpose was not fulfilled in this case, as normalization is not applicable to single cell transcriptomes. Indeed, single cell gene expression has been demonstrated to follow a lognormal distribution rather than a Gaussian distribution. As "normalization" by definition implies that the sample distributions follow a normal distribution, such statistical approaches are rendered impossible.Due to long-term difficulties with these hypothetically ubiquitous genes, primer concentration optimization was performed. In order to determine the optimal primer concentrations for low template quantities each primer was serially diluted and the non-specific amplification determined(Table 3.19. Concentrations of each primer at which minimal non-specific amplification would take place were selected. After primer optimization, standard curves were performed with serially diluted template in order to make sure that reaction efficiency was as close to 1 as possible(R2=1)(Fig. 3.18 and Fig. 3.19).

Moreover, Altincicek and Vilcinskas characterized gene expression in *Platynereis* immune cells[314]. The authors injected bacterial lipopolysaccharide, which elicit immune responses and used a subtractive hybridization technique to enrich cDNAs of differentially expressed genes. Their analysis yielded 288 cDNAs. qPCR primers were designed for arbitrarily selected immune-specific genes of *Platynereis* 3.7.

Pilot RNA characterization study Ciliated prototroch cells from larvae aged 12 and 24 hpf were isolated as described in Chapter 2, using the manual triage method. Cells were aliquoted into 0.5mL PCR tubes and cDNA was obtained using the CellsDirect kit. The possibility to obtain RNA from these cells was evaluated, by performing PCRs using primers designed against genes indicated as expressed in the prototroch in the PEPD. This assay was performed on a varying amount of cells (Table 3.8). The presence of a band as determined by a band using gel electrophoresis followed by Ethidium bromide staining is indicated as x. It is notable that all genes could not be systematically recovered from different amounts of cells. It is possible transcripts could not be identified due to RNA degradation in steps prior to reverse transcription. Indeed, putative prototroch markers were overall recovered in sets of numerous cells than in sets of few cells. As the amount of cDNA obtained from the reverse transcription was too low to be evaluated quantitatively, this hypothesis could not be measured by Nanodrop or Qbit measurements. However, this data is not conclusive and can be explained by several hypotheses. It is possible that dissociated cells no longer express transcripts encoding the cytoskeletal proteins listed above. However, the genes in question have not been extensively characterized. Their absence does not indicate that the entire transcriptome of ciliated cells is affected after dissociation and in culture. "Essential"

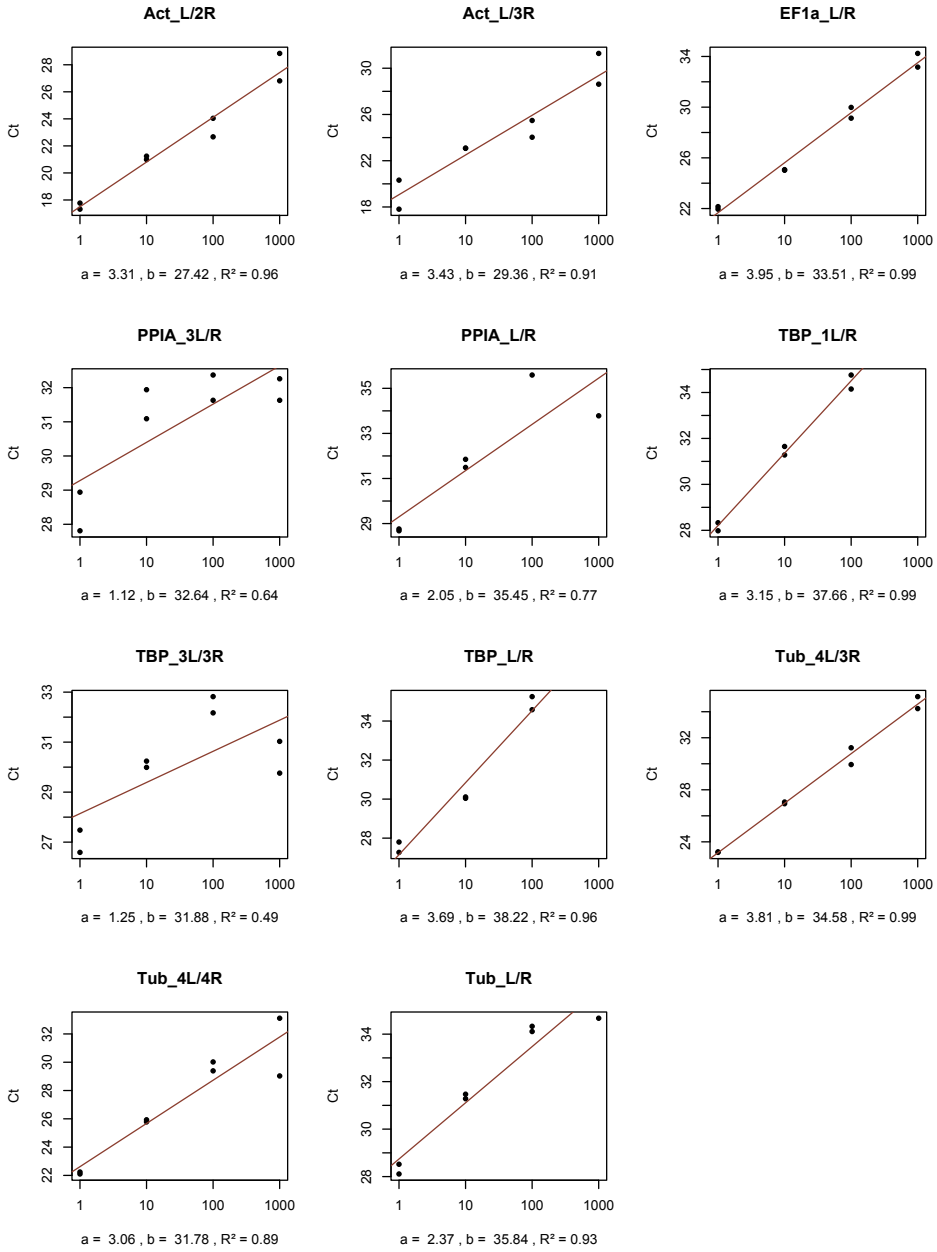


Figure 3.18: Impact of template dilution on amplification yield for interest couple of primers measured as Ct qPCR value. Adequacy is observed when product concentration decrease linearly with template concentration, and is therefore estimated using correlation coefficient R^2 obtained by linear regression.

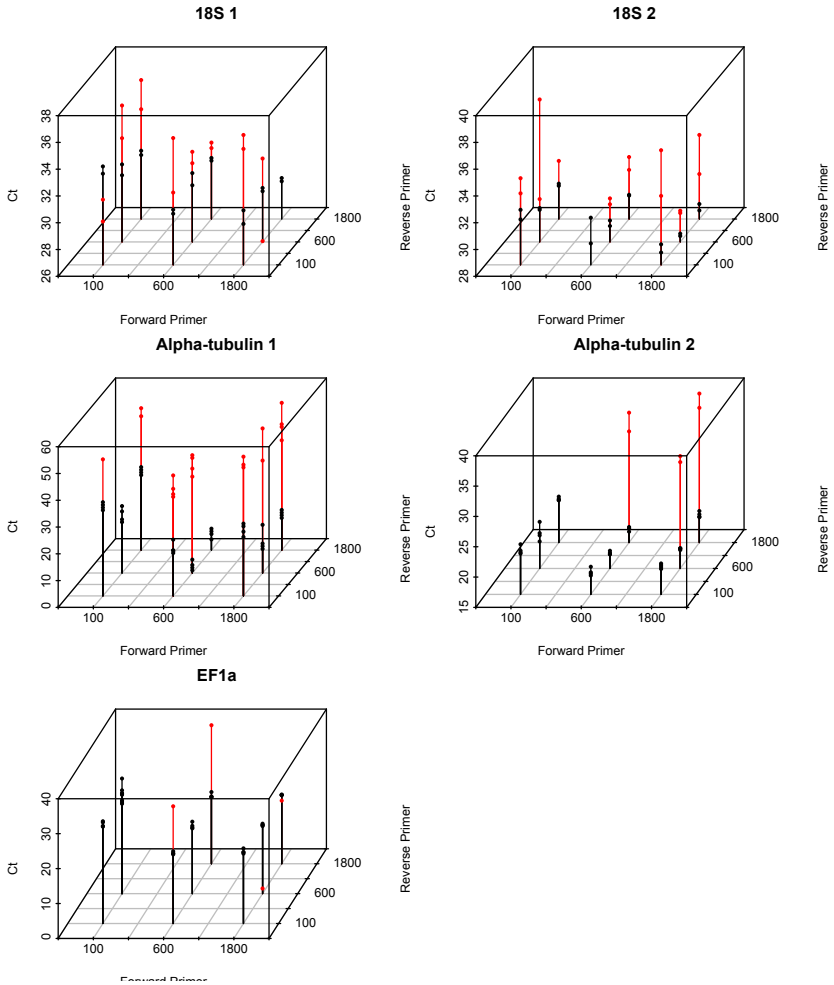


Figure 3.19: Relationship between respective forward and reverse primers concentration and amplification yield in the presence (black dots) and absence of substrate (red dots). This allows to estimate the tendency to form primer dimers and to produce non-specific amplicons.

CLONE ID	Location of expression	Name of gene
48-1-02-G	Apical tuft at 24h	Glutathione peroxidase
48-1-08-E	Apical tuft & eye at 24h	Cysteine rich protein
48-2-01-F	Prototroch at 24h	Sperm associated antigen 8
48-2-04-H	Prototroch at 24h	Mercaptopyruvate sulfurtransferase (mitochonrial)
48-2-05-C	Prototroch at 24 h	N/A
48-2-06-C	Apical tuft & prototroch at 24h	DNAJ/ Heat Shock Protein 40
48-2-08-D	Prototroch at 24h	Ornithine decarboxylase
48-2-08-E	Prototroch at 24h	Protein with ankyrin repeats
48-2-09-G	Apical tuft & Prototroch at 24h	Heat Shock binding protein 1
48-2-10-B	Prototroch at 24h	Penicillin binding protein
48-3-08-H	Apical tuft & Prototroch at 24 h	Ataxin 7
48-3-11-A	Prototroch at 24h	Histone H3
48-3-12-B	Prototroch at 24h	β -tubulin
48-5-03-F	Prototroch at 24h	Cytochrome C Oxidase subunit II
48-5-12-B	Apical tuft & Prototroch at 24h	α tubulin
631-136-02-G	Prototroch at 24h	Protein kinase C receptor
48-1-03-C	Macromeres	N/A

Table 3.5: *Platynereis dumerilii* genes specifically expressed in the prototroch based on PEPD

genes, specifying cell fate may still be expressed. Such genes have not been characterized in *Platynereis* ciliated cells. Another hypothesis may be that single *Platynereis* cells contain less RNA than mammalian and *Drosophila* cells for which this kit has been designed, thus yielding incomplete results. Moreover, primers were designed against a sequence available in the PEPD. Since cells were obtained from a heterogenous (non-inbred) population of worms, it is possible that cDNAs were not amplified due polymorphisms in the region overlapping the primers. As the goal of this study was simply to evaluate the feasability of RNA extraction and reverse transcription from few *Platynereis* larval cells, rather than the characterization of prototroch gene expression, further efforts were not put into primer design.

Gene name	Expressed in oocytes
Rpl12	+
MyoD	+
Mef2	+
FGFR	+
Troponin L	+
MRLC	+
Otx	+
Dach	+
Six3	+
Rx	+
Atonal	+
Lhx2	+
Ciliary opsin	+
Dpp	+
Pax3/7	+
Pax6	+
Sim	+
Nk6	+
Ptx	+
Hh	+
Bra	+
Smoothened	-
Sim	-
TrPH	-
Gsh	-
Mnr2	-
Dll	-
Msx	-
SoxB	-
Lhx3	-

Table 3.6: Genes shown to be expressed at 1hpf in *Platynereis* by ”absolute quantification” by Dr.Antje Fischer

Gene name
α-actinin
α-amylase
Arrestin
BPI2
Caspase2
Cub/Sushi
Defense protein 1
Dna Pol2
Ferritin H chain
Ferritin S chain
SH2
7TM
Myc
Ribosomal protein S4
NADH
Protease inhibitor
SOCS

Table 3.7: Immune-specific genes selected as candidates based on [314]

Table 3.8: Evaluation of gene expression from few cells

Name of gene	200 cells	30 cells	1 cell	5 cells	13 cells	2 cells	1 cell	19 cells	4 cells
Glutathione peroxidase	x	x							
Cysteine rich protein	x	x				x			x
Sperm associated antigen 8	x	x	x	x	x	x	x		
Mercaptopyruvate sulfurtransferase (mitochondrial)	x		x	x	x	x		x	
DNAJ/ Heat Shock Protein 40	x	x							
Ornithine decarboxylase	x				x				x
Protein with ankyrin repeats	x	x			x	x	x		
Heat Shock binding protein 1	x								
Penicillin binding protein	x		x	x				x	
Ataxin 7	x		x	x					x
Histone H3	x					x	x		
β -tubulin	x	x	x					x	x
Cytochrome C Oxidase subunit II	x	x	x	x		x	x		x
α tubulin	x			x					
Protein kinase C receptor	x						x		

Moreover, based on these results, supplementary experiments were carried out in order to identify cells with unclear morphology (Fig. 3.9). Using the same method as described above, using qPCR primers for neuronal and muscle cells, however no positive discriminatory bands were identified.

DNase treatment of isolated RNA Most RNA isolation protocols incorporate an optional DNase treatment step to digest contaminating genomic DNA (gDNA). Since DNase activity is incompatible with cDNA synthesis, inclusion of this step generally requires either subsequent RNA purification or thermal inactivation of the DNase I enzyme. RNA may be lost during the purification step and conversely heat inactivation can damage RNA integrity. Although gDNA carryover contamination may appear trivial, it may cause severe background. Most single cell mRNAs are known to be present in copies of 10. Therefore, 2 genomic copies may be indistinguishable from low abundance mRNA species. Furthermore, as gene duplication is common, presence of genomic paralogs or pseudogenes may complicate subsequent qPCR analysis. The Kurimoto/Tang/AB protocols does not implement a DNase I step. Cells are directly processed for reverse transcription. Personal communication with WTA kit manufacturer Sigma (Dr. Markus Veit and Dr. Thomas Fuhr, Markus.Veit@sial.com, Thomas.Fuhr@sial.com) recommended a DNase I digestion step, immediately followed amplification. DNase I digestion was tested in low volumes for different amounts of time: 5, 10 and 15 minutes with diluted RNA to 20, 10 and 1pg (single cell quantities) and with individual cells aliquoted into each well incubated at 25, 37 and2 $42\,^{\circ}\mathrm{C}$. DNase I was then heat inactivated either in presence of 5mM EDTA or its absence at$75\,^{\circ}\mathrm{C}$

RNA isolation from individual cells and genomic DNA removal In order to proceed to accurate gene expression profiling, nucleic acid needs to remain intact and free of proteins that could interfere with the reverse transcription process on one hand and of endogenous RNases which could be released from subcellular compartments upon cell lysis. Commercial kits can be result in considerable loss, which cannot be admitted when dealing with picogram amounts of starting material. RNA isolation kits commonly make use of chaotropic agents or lysis buffers containing detergents. Furthermore, a mixture dubbed PurAmp containing: 0.25% sarcosyl, 2 M GITC, 100 mM 2-mercapto-ethanol, 0.01 M sodium citrate, pH 7.0 (all reagents from Stratagene, La Jolla, CA), 1% (vol/vol) dimethylsulfoxide was employed by Hartshorn and colleagues [202]. Alternatively, some single cell protocols bypass the RNA isolation step, diluting the cell instead in the reverse transcription mix and performing lysis through freeze-thaw cycles. Denaturation of proteins involves incubation of the sample at high temperatures for prolonged periods of time, risking RNA degradation. As Hartshorn and colleagues described there are drawbacks to each of these approaches. On one hand the presence of denaturing chemicals impedes further analysis by denaturing reverse transcriptase and polymerase enzymes. On the other hand, methods relying on cell lysis without subsequent purification of RNA do not remove proteins that can interfere with the subsequent reactions resulting in suboptimal preparations. Mild detergent treatments can successfully remove the cell membrane without affecting the nu-

clear, which prevents analysis of nuclear-localized transcripts [202]. Guanidine isothiocyanate (GITC)is commonly used for nucleic acid extraction. Bengtsson observed complete RNase inhibition using 0.5M GITC [315]. This property allows long-term storage of samples. GITC is commonly removed by phenol-chloroform precipitation or by absorption to a solid surface linked to non-specific probes. Both these approaches have the disadvantage of being labor-intensive, time-consuming and of incurring the loss of sample [315, 202].

Platynereis larvae are free swimming and until the nectochaete stage do not consume food. Instead they rely on a series of lipid droplets containing the necessary nutrients. Lipids have been shown to interfere with RNA extraction. In order to evaluate which method is most efficient at preserving RNA in *Platynereis*larvae a comparison based on that taken up by Bengtsson and colleagues was performed. Different amounts of denaturing agents (GITC 0-0.5M, 2-mercaptoethanol 0,5-1,5%, non-chaotropic detergent Nonidet NP40 at 4% and 0,5% and in presence and absence of Proteinase K at 0,4mg/ml) were compared on *Platynereis* oocytes and 5dpf-old larvae. All samples were processed in presence of 1 μl of RNase inhibitor (Promega). Cell lysis was attempted at 60 and 80 °C. Lysed samples were directly processed for reverse transcription using random hexamer and oligo(dT) primers (Fig. 3.20). The result of the reverse transcription was purified using QIAGEN RNeasy columns and quantified using a Nanodrop. In absence of GITC, 1,5% 2-mercaptoethanol produced the best results. At 40mM GITC, all solutions containing NP-40 performed best. At 80mM GITC NP-40 and PK samples performed best. Whereas at 500mM addition of Proteinase K had no positive effect. Instead these samples performed least well. Furthermore, extraction of RNA followed by reverse transcription in the same conditions from single cells using Qiagen's RNeasy columns, Trizol (Invitrogen), using Lysis Buffer from the CellsDirect One-Step qPCR kit (Sigma) and homemade PurAmp did not yield positive results, no qPCR amplification was observed and Nanodrop readings were "negative" or lower than 3-4ng which is within the error margin of the instruments measurements. Indeed Methods such as Trizol and RNeasy mini kit that involve multiple transfer steps can result in loss of material. Although the Lysis Buffer from the CellsDirect kit was efficient with the CellsDirect Kit which makes use of Sigma's Superscript III reverse transcriptase, it did not perform well using the same enzyme and a generic reverse transcription buffer. Indeed, the CellsDirect kit, unlike standard reverse transcription kits from Sigma utilizing Superscript III makes use of a different buffer, the 5x reverse transcription buffer. High salt concentrations are known to be good at preserving RNA integrity, but also inhibit a number of enzymatic reactions. Although the composition of the Lysis buffer is not known, it is possible that the cumulative salt concentration from the seawater in which the sample is prepared, the lysis buffer and those in the reverse transcription buffer used may have been inhibitory to the reverse transcription reaction. Although, PurAmp was been successful at preventing degradation of RNA from single cells, in my hands, this method did not work. Results were quantified using the Nanodrop and via qPCR for rpl12 gene. Extractions were carried out at 60 and 80 °C. Reactions carried out at 80 °C clearly were more efficient, unless if used in combination with Proteinase K, which is likely denatured at this temperature. Based on these results, a subsequent study was carried out on older larvae (5days old) to verify that such tough conditions are not simply a prerequisite to removing the tough chorion

present in the egg 3.21.

RNase inhibitors All these tests were carried out in presence of RNase inhibitors. Attempts to carry out reactions in the complete absence of RNase inhibitors supplemented at any step systematically failed. The most commonly employed RNase inhibitor in the Arendt lab is ScriptGuard RNase Inhibitor(Epicentre) for the purpose of *in situ* hybridization probe production and *in vitro* transcription. Yabuta and colleagues employed RNAguard RNase Inhibitor (GE Healthcare) [273] and Kurimoto and colleagues used RNAguard RNase Inhibitor (GE Healthcare) in conjunction with Prime RNase Inhibitor (Eppendorf). ScriptGuard RNase Inhibitor, Prime RNase Inhibitor and RNAguard RNase Inhibitor can only bind to RNase A-type enzymes such RNases A, B and C, but do not affect RNases T1, T2, H, U1, U2 or CL3. Tang and colleagues employed two different more expensive RNase inhibitors Rnasin (Promega, Catalogue number N2611) and SUPERase-In(Ambion, Catalogue number AM2694) [316]. Soon after I started these experiments Prime RNA inhibitor ceased to be distributed by Eppendorf. SUPERaseIn has several advantages over RNase inhibitors derived from mammalian RNase Inhibitor, aside from inhibiting only type A RNases, only function up to $40\,^{\circ}\mathrm{C}$ and require the presence of DTT to bind RNases. SUPERase-In binds RNase T1 and RNase 1 as well as RNase A, B, C is active at temperatures reaching $65\,^{\circ}\mathrm{C}$ and does not require DTT. RNasin binds type A RNases but has the advantage of remaining active for up to 15 minutes at $70\,^{\circ}\mathrm{C}$. These properties allow these RNase inhibitors to remain active during the DNase I heat inactivation step at 75 degrees, further preserving RNA integrity. In an experiment utilizing the WTA (Sigma) amplification method described below, in the presence of SUPERase-In and RNasin cDNA yields were higher than yields obtained using Scriptguard RNase inhibitor(Table 3.9. To investigate this aspect further, mammalian RNase A sequences (Expasy ENZYME entry: EC 3.1.27.5) were blasted against the genomes of *Capitella*, *Lottia*, *Branchiostoma* and the preliminary draft genome of *Platynereis*. No sequences were uncovered that upon reverse blasting could yield RNase A sequences.Based on this observation, one can conclude that *Platynereis* cells do not endogenously express type A ribonucleases. Whether the cells manipulated in this study indeed express another class of ribonucleases. Supplementing single cell RNA amplification studies with RNase inhibitors specific for mammalian RNases would only inhibit RNases carried over from manipulators, but would not inhibit endogenous ribonucleases. As SUPERase-In has a broader range of specificity, it may be able to inhibit endogenously expressed ribonucleases. Indeed among targets of SUPERase-In is RNase T1. RNase T1 was indeed observed among the annotations of the other Lophotrochozoan genomes available. Identification of gene sequences in genome resources of course does not demonstrate that gene products of these genes are expressed in *Platynereis* cells, but does give an indication.

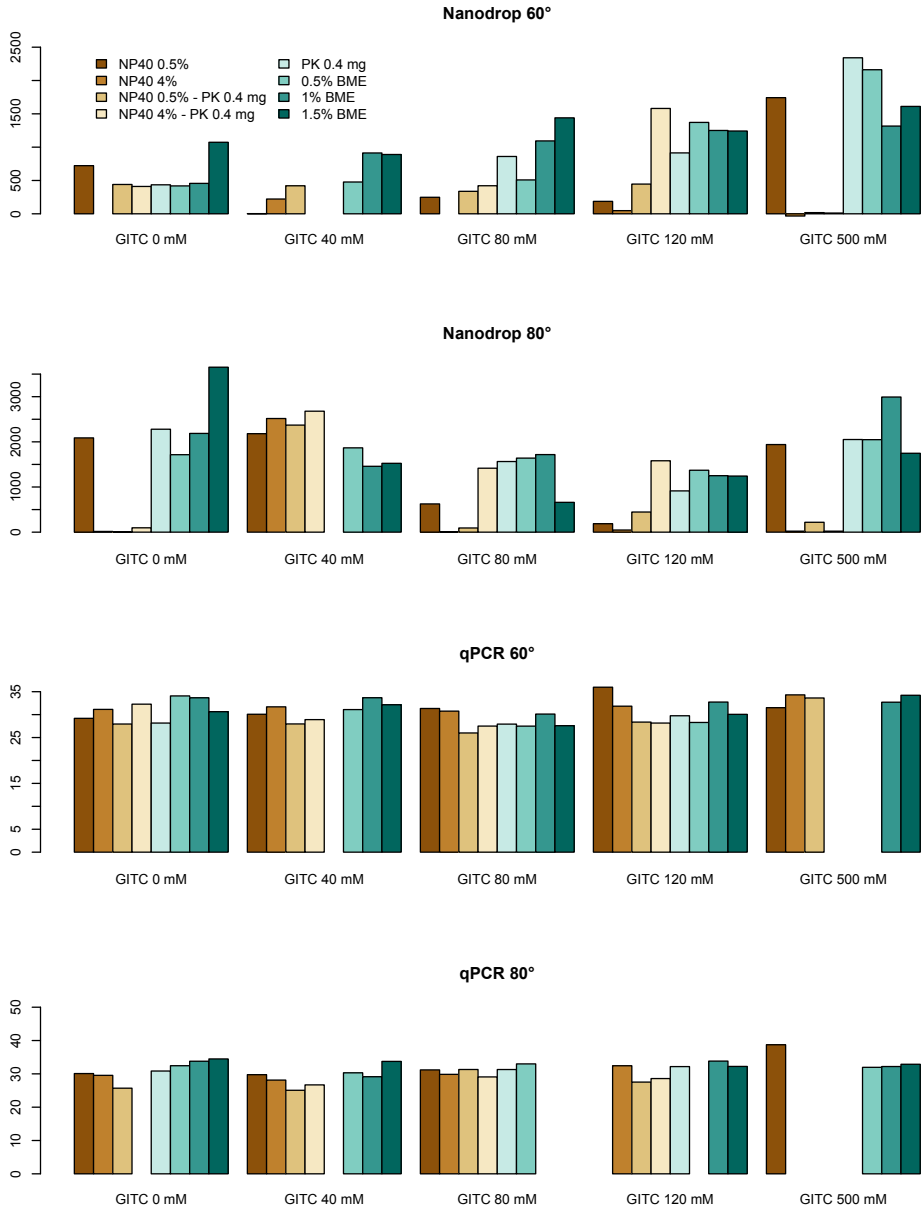


Figure 3.20: RNA extraction from individual oocytes in presence of chaotropic and non-chaotropic reagents:NP40 0.5%, NP40 4%, NP40 0.5% - Proteinase K 0.4 mg/ml, NP40 4% - Proteinase K 0.4 mg/ml, Proteinase K 0.4 mg/ml, 0.5% BME, 1% BME, 1.5% BME

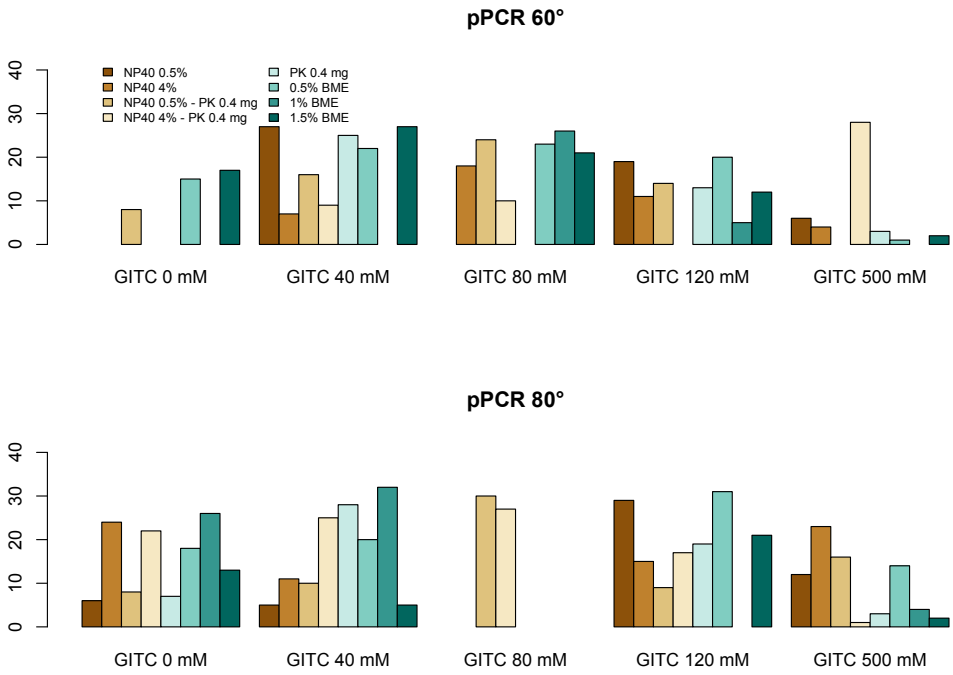


Figure 3.21: RNA extraction from individual larvae in presence of chaotropic and non-chaotropic reagents:NP40 0.5%, NP40 4%, NP40 0.5% - Proteinase K 0.4 mg/ml, NP40 4% - Proteinase K 0.4 mg/ml, Proteinase K 0.4 mg/ml, 0.5% BME, 1% BME, 1.5% BME

RNase inhibitor employed	Sample	Concentration (ng/μl)	A260	A280	260/280	260/230	340 raw
Scriptguard	1-1	8.545 +/- 1.223294731	0.171 +/- 0.024041631	0.0975 +/- 0.010606602	1.75 +/- 0.0035	2.33 +/- 0	0.0295 +/- 0.054447222
Scriptguard	1-2	11.41 +/- 0.127279221	0.228 +/- 0.002828427	0.119 +/- 0.011313708	1.925 +/- 0.0605	1.875 +/- 0.021213203	0.147 +/- 0.14707821
Scriptguard	1-3	21.53 +/- 0.240416306	0.4305 +/- 0.004949747	0.2345 +/- 0.019091883	1.845 +/- 0.03	1.135 +/- 0.06363961	0.1265 +/- 0.010606602
Scriptguard	1-4	26.24 +/- 0	0.5315 +/- 0.009192388	0.317 +/- 0.012727922	1.68 +/- 0.143	1.03 +/- 0.028284271	0.227 +/- 0.001414214
Scriptguard	1-5	27.245 +/- 36.94632932	0.5445 +/- 0.738926586	0.338 +/- 0.446891486	1.33 +/- 0.04	0.455 +/- 0.403050865	0.8365 +/- 0.156270599
Scriptguard	1-6	32.56 +/- 29.42978423	0.651 +/- 0.588312842	0.378 +/- 0.390322943	1.97 +/- 0.237	1.595 +/- 1.209152596	0.359 +/- 0.519016377
Scriptguard	1-7	13.55 +/- 2.545584412	0.271 +/- 0.050911688	0.141 +/- 0.055154329	2.005 +/- 0.0725	2.19 +/- 0.367695526	0.0255 +/- 0.047376154
Scriptguard	1-8	39.325 +/- 7.43169227	0.7865 +/- 0.149199531	0.466 +/- 0.089095454	1.69 +/- 0.0515	0.965 +/- 0.049497475	0.397 +/- 0.041012193
Scriptguard	1-9	44.23 +/- 0.494974747	0.885 +/- 0.009899495	0.5405 +/- 0.016263456	1.64 +/- 0.1925	0.92 +/- 0.113137085	0.4575 +/- 0.044547727
Scriptguard	1-10	59.65 +/- 22.30214788	1.193 +/- 0.445477272	0.756 +/- 0.288499567	1.58 +/- 0.1315	0.77 +/- 0.098994949	0.8525 +/- 0.51406663
Scriptguard	1-11	49.7 +/- 36.37357282	0.994 +/- 0.726905771	0.6245 +/- 0.47446865	1.615 +/- 0.26	0.845 +/- 0.205060967	0.7085 +/- 0.717713383
Scriptguard	1-12	18.005 +/- 8.449926035	0.3605 +/- 0.168998521	0.2135 +/- 0.106773124	1.705 +/- 0.4315	1.37 +/- 0.537401154	0.108 +/- 0.131521861
Scriptguard	1-13	12.44 +/- 0.579827561	0.249 +/- 0.011313708	0.645 +/- 0.717006276	1.72 +/- 0.0545	1.8 +/- 0.070710678	0.026 +/- 0.015556349
Scriptguard	2-1	17.13 +/- 4.737615434	0.343 +/- 0.094752309	0.1815 +/- 0.092630988	2.02 +/- 0.0615	1.58 +/- 0.480832611	0.078 +/- 0.04384062
Scriptguard	2-2	13.745 +/- 0.049497475	0.275 +/- 0.001414214	0.12 +/- 0.005656854	2.3 +/- 0.1845	1.995 +/- 0.106066017	0.0445 +/- 0.003535534
Scriptguard	2-3	29.69 +/- 22.59913273	0.5935 +/- 0.451841233	0.3125 +/- 0.266579257	2.02 +/- 0.32	2.155 +/- 0.120208153	0.0335 +/- 0.012020815
Scriptguard	2-4	91.075 +/- 64.2123668	1.8215 +/- 1.284813021	1.0095 +/- 0.719127596	1.81 +/- 0.0085	1.92 +/- 0.45254834	0.2595 +/- 0.33163308
Scriptguard	2-5	86.735 +/- 70.35005366	1.735 +/- 1.407142495	1.001 +/- 0.731148412	1.665 +/- 0.4305	1.195 +/- 0.572756493	0.494 +/- 0
Scriptguard	2-6	45.625 +/- 12.21173411	0.9125 +/- 0.24395184	0.5705 +/- 0.122329473	1.59 +/- 0.206	0.81 +/- 0.028284271	1.563 +/- 1.511794298
Scriptguard	2-7	13.145 +/- 11.17935821	0.263 +/- 0.223445743	0.152 +/- 0.113137085	1.615 +/- 0.016	1.09 +/- 0.636396103	0.7175 +/- 0.744583441
Scriptguard	2-8	34.645 +/- 29.8328351	0.693 +/- 0.596798123	0.3885 +/- 0.402343758	2.135 +/- 0.2705	114.57 +/- 160.4142444	297.51 +/- 420.7143927
Scriptguard	2-9	56.5 +/- 1.074802307	1.13 +/- 0.021213203	0.687 +/- 0.01979899	1.645 +/- 0.072	1.15 +/- 0.014142136	297.795 +/- 420.3113418
Scriptguard	2-10	112.835 +/- 90.16318567	2.257 +/- 1.803122292	1.2395 +/- 1.004798736	1.83 +/- 0	1.9 +/- 0.028284271	0.285 +/- 0.287085353
Scriptguard	2-11	176.59 +/- 0	3.532 +/- 0	1.95 +/- 0	1.81 +/- 0	1.88 +/- 0	0.488 +/- 0
Superas-in & Rnasin	a	21.475 +/- 7.106423151	0.4295 +/- 0.142128463	0.2285 +/- 0.092984542	1.885 +/- 0.074246212	4.035 +/- 0.982878426	-0.003 +/- 0.166170094
Superas-in & Rnasin	a	31.525 +/- 7.51300955	0.6305 +/- 0.150260191	0.36 +/- 0.098641396	1.78 +/- 0.06363961	2.645 +/- 1.103086579	0.232 +/- 0.163341666
Superas-in & Rnasin	b	42.15 +/- 5.982123369	0.843 +/- 0.119854599	0.4995 +/- 0.060104076	1.69 +/- 0.024748737	1.085 +/- 0.240416306	0.463 +/- 0.063993164
Superas-in & Rnasin	b	50.61 +/- 6.204862005	1.0125 +/- 0.12409724	0.5845 +/- 0.060811183	1.725 +/- 0.031819805	1.425 +/- 0.254558441	0.3725 +/- 0.070357125
Superas-in & Rnasin	c	59.385 +/- 0.74246212	1.188 +/- 0.014849242	0.6705 +/- 0.012727922	1.77 +/- 0.053033009	1.785 +/- 0.240416306	0.273 +/- 0.479064844
Superas-in & Rnasin	c	58.335 +/- 0.088388348	1.167 +/- 0.001767767	0.6885 +/- 0.027930718	1.695 +/- 0.060104076	1.445 +/- 0.258093975	0.9505 +/- 0.497096067
Superas-in & Rnasin	d	58.46 +/- 2.032931996	1.1695 +/- 0.04065864	0.728 +/- 0.026870058	1.61 +/- 0.003535534	1.08 +/- 0.028284271	1.6535 +/- 0.161220346
Superas-in & Rnasin	d	61.335 +/- 7.258451109	1.227 +/- 0.145310444	0.766 +/- 0.113844192	1.605 +/- 0.08131728	1.04 +/- 0.395979797	1.4255 +/- 0.580534667
Superas-in & Rnasin	e	51.07 +/- 7.102887617	1.0215 +/- 0.142128463	0.605 +/- 0.101823376	1.72 +/- 0.045961941	1.6 +/- 0.403050865	0.6045 +/- 0.390676497
Superas-in & Rnasin	f	41.025 +/- 3.351686143	0.8205 +/- 0.067175144	0.461 +/- 0.039951533	1.785 +/- 0.010606602	2.17 +/- 0.187383297	0.052 +/- 0.013081475
Superas-in & Rnasin	f	45.765 +/- 3.408254685	0.9155 +/- 0.068235804	0.5175 +/- 0.030052038	1.77 +/- 0.024748737	2.435 +/- 0.197989899	0.0335 +/- 0.010960155
Superas-in & Rnasin	g	50.585 +/- 0.703571247	1.012 +/- 0.014142136	0.56 +/- 0.003535534	1.805 +/- 0.010606602	2.715 +/- 0.003535534	0.018 +/- 0.011313708
Superas-in & Rnasin	g	51.58 +/- 0	1.032 +/- 0	0.565 +/-0	1.82 +/- 0	2.71 +/- 0	0.034 +/- 0

Table 3.9: Comparison of cDNA yield using WTA kit (Sigma) with different RNase inhibitors

Table 3.10: Ribonucleases uncovered in the genome of *Capitella telata*(JGI)

InterPro Domain	Description	Number of protein models	Protein IDs
IPR003029	RNA binding S1	2	175688.1607
IPR001568	Ribonuclease T2	1	154023
IPR000999	Ribonuclease III	3	222168,165112,223153
IPR001352	Ribonuclease HII/HIII	1	183401
IPR002114	HPr serine phosphorylation site	1	197082
IPR005987	Ribonuclease T	1	142825
IPR007087	Zn-finger, C2H2 type	1	227646
IPR002156	RNase H	22	101318,213104,217934,222606,189741,...
IPR002562	3'-5' exonuclease	1	189986
IPR006292	Ribonuclease D	1	189986
IPR003100	Argonaute and Dicer protein, PAZ	1	223153
IPR004664	Ribonuclease BN	1	213855
IPR005135	Endonuclease/exonuclease/phosphatase	2	217934.2063
IPR002759	Ribonuclease P-related protein	2	219067.1506
IPR006641	Ribonuclease RNase H fold containing	3	59334,175688,160659
IPR001900	Ribonuclease II	4	108980,225692,225282,227646
IPR006055	Exonuclease	1	142825

RNA Amplification

Modulating Reverse Transcription In order to increase the efficiency of the reverse transcription of small quantities of transcript, a protocol described by Tougan and colleagues was tested [248, 246]. The authors indicate that enzymes following Michaelis-Menton kinetics are inefficient in presence of low substrate concentrations. Therefore, they propose spiking the reaction with an unspecific substrate dubbed "Chum RNA" in order to increase the global RNA concentration. Chum RNA was added to reverse transcription reactions using the protocol described by Tougan and colleagues, using the Qiagen Quantitect Reverse Transcription Kit and the Kurimoto reverse transcription reaction. (Fig. 3.22)Supplementing reverse transcription reactions that used 1ng/μl Affymetrix spike-in controls as template with 10 to 0 μg Chum RNA did not appear to have a dose-dependent effect. A 1000 bp product was observed in all reactions containing Chum RNA. This product was absent in the No Template and No Reverse Transcriptase controls, indicating that the 1000bp band is the result of reverse transcription and requires the presence of both template, enzyme and Chum RNA(Fig. 3.22A). Supplementing reverse transcription reactions containing both mRNA and spike-in controls (25pg/μl each) with 10 to 0 μg Chum RNA yielded a similar side band. Faint smears were also present, whether they correspond to reverse transcribed mRNA was difficult to judge Fig. 3.22B). In order to ascertain that Chum RNA is universally useful as a supplement to reverse transcription reactions, an independent protocol - the Qiagen Quantitect reverse transcription kit was supplemented with 10 to 0 μg Chum RNA. Similarly, using Affymetrix spike-in controls at 1ng/μl as template an approximately 1000bp product was observed in all samples containing Chum RNA, although clear dose-specific effect was not observed(Fig. 3.22C).However, when performing the same reactions supplemented with Chum RNA on total RNA extracted from mature adult *Platynereis*, smears were observed in all samples containing Chum RNA but the 1000bp product was absent(Fig. 3.22C).

Attempts at linear amplification of *Platynereis* single cell derived-RNA Linear amplification first developed by Van Gelder, Eberwine, and coworkers is an alternative to PCR that unlike exponential protocols does not induce a bias during the amplification procedure. Linear amplification was carried out using the primers and solutions proposed by Nojima and Tougan [248, 246] without addition of Chum RNA to determine whether two-cycle linear amplification is sufficient to produce large enough amounts of amplicon for subsequent analysis. This test was performed using Affymetrix spike-in controls. cDNA yield was quantified by using a Nanodrop and relative gene expression was assay via qPCR. Nanodrop quantification gave values within the error margin of the Nanodrop. No sufficient amplification was observed using qPCR of spike-in controls.

Attempts at amplification of *Platynereis* single cell derived-RNA using Kurimoto-Tang Several protocols have been published for the characterization of mouse blastomeres based on a protocol originally described by Kurimoto and colleagues. Preliminary testing was performed using purified mRNA, total RNA and Affymetrix (R) Spike-in control RNAs as well as with single isolated cells. Aliquotes of 0.5μl were

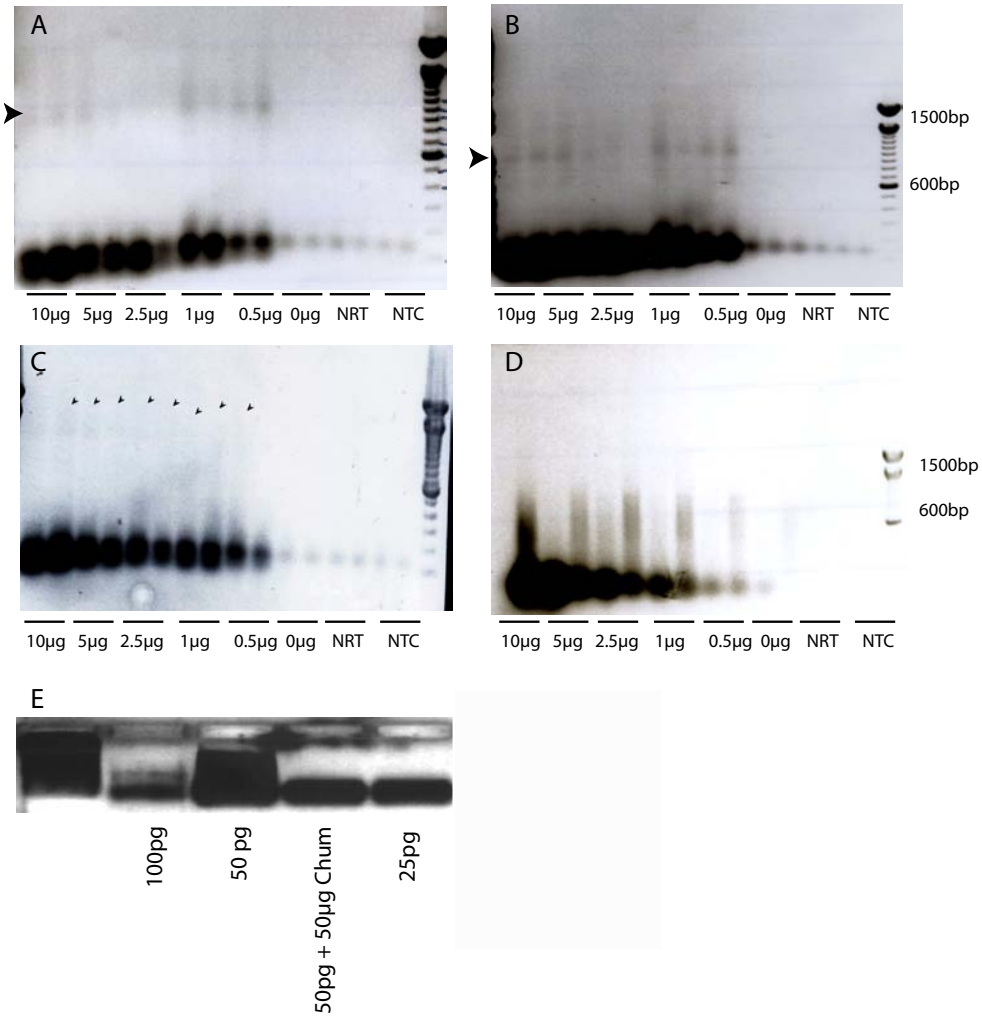


Figure 3.22: Effect of spiking RT reactions with 10, 5, 2.5, 1, 0.5 and 0 g Chum RNA. Reverse transcription with Superscript III (A)using spikes only and (B) using spikes (1ng/μl) and mRNA; Reverse transcription with Qiagen Quantitect RT Kit (C)using spikes only and (D)spikes and mRNA. (E)Effect of 5 micrograms of Chum RNA spiking using Kurimoto amplification procedure, black arrows indicate the 1000bp

taken at each step. PCRs were performed on each sample to confirm the presence of ubiquitous genes and selected marker genes. (Table . 3.11) However, results were invariably not replicable between different stages of the amplification procedure.

Table 3.11: Evaluation of single cell amplification protocol (Kurimoto-Tang)

	8	16	8	8	8	8	8	32
Name of gene/Nb. Of cells								
Glutathione peroxidase	x	x	x					x
Cysteine rich protein								
Sperm associated antigen 8				x	x		x	
Mercaptopyruvate sulfurtransferase (mitochondrial)								
DNAJ/ Heat Shock Protein 40		x						
Ornithine decarboxylase			x					
Protein with ankyrin repeats						x		x
Heat Shock binding protein 1	x			x				
Penicillin binding protein		x						
Ataxin 7								
Histone H3								x x
β-tubulin								
Cytochrome C Oxidase subunit II		x				x		
α tubulin							x	
Protein kinase C receptor								x
Thr (spike-in control)	(x)	(x)		x	x		x	x
Dap (spike-in control)	(x)		(x)	x		x		
Lys (spike-in control)	(x)	(x)						
Phe (spike-in control)	(x)							

Sample	Concentration (ng/ul)	A260	A280	260/280	260/230	340 raw
Non-diluted primers sample 1	282.88	5.658	3.093	1.83	3.22	0.017
Diluted primers sample 1	314.48	6.290	3.447	1.82	3.23	0.034

One explanation for the absence of replicability of this experiment and of the linear amplification method may be the salts present in the seawater in which the cells are incubated. Salts are well known to stabilize RNAs, however, they can also inhibit numerous enzymes (including the Superscript III, Exonuclease I, TdT and Taq polymerase which are used in these protocols). In the absence of a denaturing step at the beginning of the RNA isolation carry-over seawater may impead any subsequent reactions. In my hands both the linear amplification protocol, the protocol described by Kurimoto and the protocol described by Tang were unsuccessful. For this reason, an alternative method was tested. A recently developed commercial kit(Sigma) was put to use.

Attempts at amplification of *Platynereis* single cell derived-RNA using Whole Transcriptome Amplification Kit (Sigma) Roca-Gonzalez and colleagues described an increase in relative gene ratio preservation by diluting primers used for the reverse transcription step. This was tested by using 2 samples - one with non-diluted samples and one with diluted samples. Indeed cDNA resulting from the diluted primer reaction gave greater yield as determined by Nanodrop examination. Using dilutions of adult mRNA purified from 13 heads of mature *Platynereis* purified using Dynabeads. Amplification was attempted using dilutions of starting material to 1ng, 100pg, 10pg 0,1pg. The resulting cDNA was evaluated using the Nanodrop. As recommended by the manufacturer the appropriate number of PCR cycles necessary for amplification of low amounts of starting material by adding ROX and SYBR to the PCR reaction and observing amplification efficiency by qPCR. According to the manufacturer's instructions, PCR should be allowed to proceed 2 cycles past the plateau phase of the PCR reaction.

Roca-Gonzalez and colleagues claimed that 23 cycles of PCR were sufficient. Indeed, similar results were obtained using 1 and 10 cells. Using 100 cells plateau was attained by 12 cycles. As these results were judged encouraging, QC qPCR (Fig. 3.23) was carried out on samples obtained using this method. Using the qPCR primers listed above. Unfortunately, most of the genes tested did seem to be expressed.

Table 3.12: Quantification of whole transcriptome amplification products by spectrophotometry using staggered dilutions.

Sample	Concentration	A260	A280	260/280	260/230	340 raw
1 ng (1)	68,350 +/- 0,354	1,367 +/- 0,007	0,823 +/- 0,004	1,660 +/- 0,000	1,270 +/- 0,000	0,036 +/- 0,008
1 ng (2)	59,885 +/- 1,322	1,198 +/- 0,026	0,720 +/- 0,035	1,665 +/- 0,049	1,365 +/- 0,021	-0,059 +/- 0,023
100 pg (1)	59,945 +/- 0,658	1,150 +/- 0,071	0,710 +/- 0,029	1,695 +/- 0,049	1,565 +/- 0,049	-0,103 +/- 0,006
100 pg (2)	59,800 +/- 1,754	1,196 +/- 0,036	0,714 +/- 0,025	1,675 +/- 0,007	1,485 +/- 0,064	0,019 +/- 0,010
10 pg (1)	86,330 +/- 1,527	1,727 +/- 0,030	1,071 +/- 0,013	1,615 +/- 0,007	1,115 +/- 0,007	0,368 +/- 0,002
10 pg (2)	51,810 +/- 4,808	1,036 +/- 0,097	0,632 +/- 0,059	1,640 +/- 0,000	1,865 +/- 0,163	-0,116 +/- 0,168
0,1 pg (1)	49,355 +/- 2,143	0,987 +/- 0,042	0,574 +/- 0,029	1,720 +/- 0,014	2,530 +/- 0,071	-0,362 +/- 0,017
0,1 pg(2)	46,070 +/- 0,226	0,922 +/- 0,005	0,538 +/- 0,003	1,710 +/- 0,000	2,640 +/- 0,014	-0,345 +/- 0,018

3.3 Discussion

Morphological characterization of *Platynereis* early trochophore larval cells The vast majority of *Platynereis dumerilii* cells obtained by Pronase dissociation were quasi-indistinguishable one from another. Although within the context of the animal cells take up certain morphologies due to pressure from their surroundings, once this pressure is release cells take up a slightly squashed spherical appearance. Based solely on morphology, cells could be separated in to few groups, based on size. Although at the stages examined (24-48 hours) larvae are endowed with muscle cells, no spontaneously contracting muscle-like cells were observed. This may be due to the fact that there are quite few of these cells. At 48hours muscle-dependent movements are observed in the larva. Perhaps at this stage cells do not spontaneously contract as is the case with older muscle cells. Based on the observations carried out in the present study isolation of living cells based purely on morphology is possible with very few cell types. The only cells that could be accurately distinguished from their counterparts were ciliated cells, pigmented cells and macromeres. If any form of single cell transcriptome characterization were to be carried out on larvae obtained from these stages, expression of fluorescent reporter genes would be required.

Morphological characterization of *Platynereis* ciliated larval cells *Platynereis* ciliated cells were among the few cell types that could be readily recognized in a mixture of cells obtained from dissociated larvae. These cells "swim" in a calcium concentration-dependent fashion. Their movement does not appear to be random. Some cells appear to swim in concentric trajectories, whereas other cells appear to move in trajectories that are practically linear. Hypotheses explaining this phenomena were proposed. The difference in swimming trajectory could be an artifact of the dissociation process, the proteolytic treatment affecting the cilia of some cells, resulting if failure to move around in different ways. This theory is however contradicted by the observation that if embryos are dissociated at stages prior to trochophore formation and ciliogenesis takes place in already dissociated cells, the difference in swimming pattern is still observed. This indicates that the different swimming behaviors are inherent to the cells themselves rather than to manipulation. An alternative hypothesis is that cells could be less likely to displace themselves as rapidly if they are simply larger and thus "heavier" to transport. This theory is annulled by a comparison of cells sizes, demonstrating that cell size does not correlate with swimming behavior. Furthermore, incomplete dissociation sometimes results in cells being attached to another cell. Swimming ciliated cells with "a passenger" were not less likely to move in concentric movements that those without. Thus it would appear that there are two cell populations present as early as the appearance of the first prototroch cells (12-15hpf) that behave in different fashions. The straight-swimming cells are in a minority compared to concentrically swimming cells. It is tempting hypothesize that these cells are intrinsically different at their origin, and to compare them to the two "stearing cells" observed by Jekely and colleagues which set the example to the other ciliated cells in the prototroch. The stearing cells are directly innervated by the larval photoreceptors, thus receiving directional information directly from the eyes. Jekely and colleagues furthermore, demonstrated

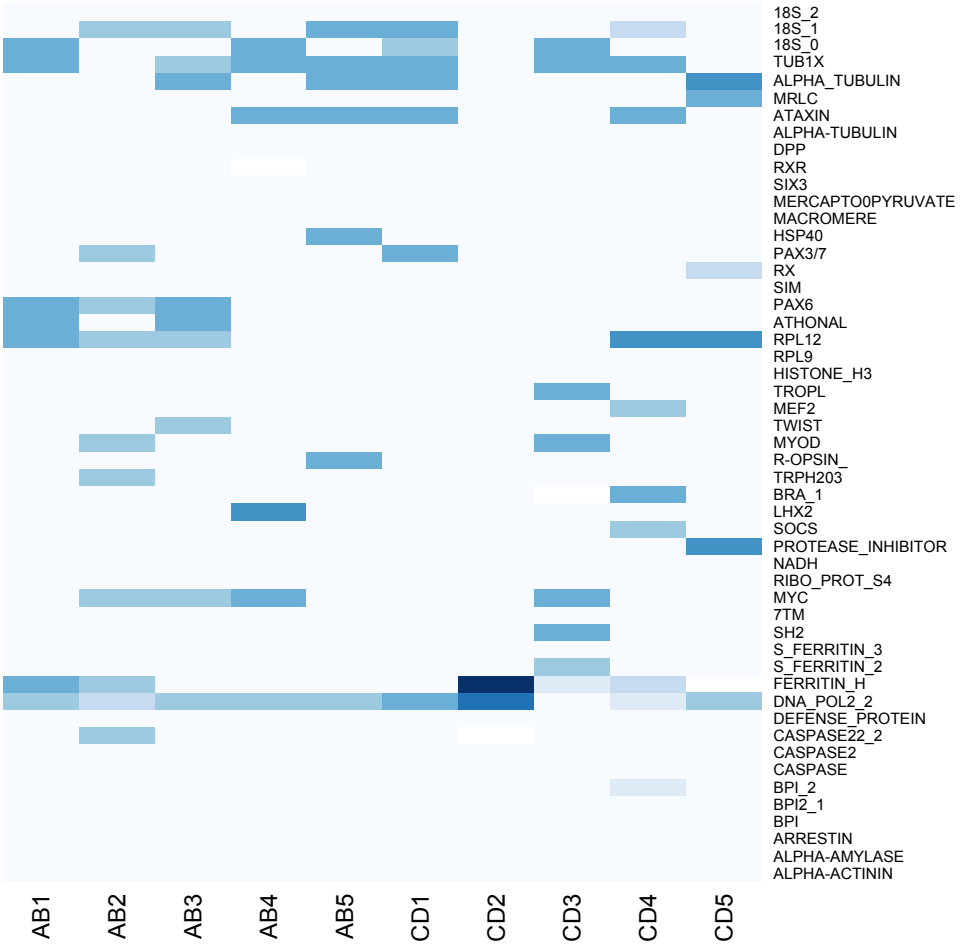


Figure 3.23: Heatmap representation of expression levels for candidate quality control genes by qPCR. Lower labels correspond to blastomere identity and sample id.

that photoreceptor projections innervating the stearing cells were present. Treatment of dissociated cell culture with exogenous mimicking neurotransmission did not affect swimming behavior. Among several compounds tested, only an α adrenergic receptor agonist affected ciliary beating, in line with data from vertebrate studies. In conclusion, at least two ciliated cell populations are present in the prototroch whose behavior *in vitro* is strikingly different and does not respond to drug treatment and does not correlate to their morphology. As ciliated cells were observed to swim faster in presence of higher concentrations of calcium, it may also be possible that micro-scale local differences in calcium concentrations are responsible for more rapid ciliary beating resulting in different form of locomotion. Incubation of ciliated cells in media containing higher overall calcium concentrations (DMEM) and lower over calcium concentrations (Schneider) did not affect the appearance of both types of swimming behavior.

Morphological characterization of *Platynereis* adult cells Cells obtained from adult *Platynereis* were much more numerous. These cells were often contaminated by large unicellular organisms. This can be explained by their original presence in the gut of the animal and subsequent liberation upon dissociation. Most of the cells observed from the coelomic fluid corresponded to elaeocytes. These cells appeared to be surrounded by large shiny viscous lipid-like substance. Attempts made to obtain *Platynereis* nerve cells were unsuccessful. Adult muscle cells were readily recognizable. Two populations of cells were observed those that "twitched" and those that "contracted". It was hypothesized that the first group corresponded to striated muscle whereas the second group corresponded to gut smooth muscle. Contraction speed could be quantified. *Platynereis* adult cells unlike larval cells demonstrated extreme variability in color and morphology.

Pilot study in molecular characterization of *Platynereis* ciliated cells A pilot study using a commercially distributed kit was carried out in order to determine whether cDNA could be obtained from *Platynereis*dissociated cells. Swimming prototroch ciliated cells were selected. Based the *Platynereis* Expression Pattern Database, primers were designed against genes known to be specifically expressed in ciliated cells. The pilot study demonstrated that mRNA from individual *Platynereis* dissociated ciliated cells could be reverse transcribed into cDNA and that expression could be assayed by qPCR. Although, not all markers tested were systematically present in all cells this preliminary test was judged sufficient to confirm that subsequent to the dissociation procedure described in Chapter 2, RNA could be obtained reliably enough to perform reverse transcription and that PCR products could be obtained from the obtained cDNA. Indeed, carryover pronase could result in the enzymatic destruction of DNase I, reverse transcriptase and taq polymerase enzymes. This was not observed. Therefore, further efforts were invested into the attempts to obtain cDNA from single or few cells from *Platynereis* larvae.

Attempts at single cell amplification Several methods were attempted in order to obtain cDNA from individual or groups of few pooled cells. Linear amplification protocols and the Kurimoto [274] protocol were tested and were unsuccessful. Despite

numerous attempts, replicable results were not obtained whether on single or few cells, using ciliated prototroch cells. This is unlike the results obtained using the CellsDirect Kit with which although RNA degradation was likely to have been present in samples containing few cells, PCR products of marker genes could be obtained. The protocols linear and kurimoto protocols involved lysis of cells in RT medium. The only denaturing agent present was a detergent. Whereas, in the CellsDirect Kit, a denaturing lysis buffer was utilized. Furthermore, the Kurimoto and linear protocol utilize higher volumes of starting material. Therefore, several hypotheses could be proposed to explain this discrepancy. First of all, that a cellular component present in *Platynereis* cells such as an endogenous RNase may be responsible for RNA degradation. Furthermore, an exogenous agent present in the medium present in higher amounts in the cell sample could be responsible for the lack of success. The most likely candidate could be the salt present in the seawater. High salt concentrations are known to inhibit numerous enzymatic reactions. It is of course also possible that as both the linear and Kurimoto protocols require numerous steps prior to amplification, that pipetting error or RNA contamination could have occurred. As samples were suspected to contain such small amounts of nucleic acid that aliquotes could not be taken systematically at each step in order to ascertain quality of nucleic acid. For this reason a denaturing method (Fig. 3.20) was developed for the aliquoting of cells. This method was utilized in the isolation of nucleic acids from blastomeres. This denaturing solution containing GITC and Nonidet Np-40. The best results in terms of yield were obtained using this denaturing solution, DNase I treatment and the Whole Transcriptome Amplification Kit. qPCR-based evaluation yielded dissappointing results. Few genes were observed to be expressed in large quantities. This does not necessarily indicate that RNA quality was affected by nucleic acid preparation and amplification. The genes tested correspond for the most part to transcription factors, which are generally expressed at low amounts. qPCR primers available in the lab for developmental studies were also used. However, in an ideal case Quality control primers should correspond to genes expressed at both high and low concentrations. In order to have such primers it would have been useful to first obtain a transcriptome of whole non-dissociated oocytes. Based on this data, candidate quality control primers could have been designed and tested. Usage of primers against genes expressed at low copy number or testing of arbitrary qPCR primers may result in missing key genes necessary for ambitious molecular studies such as this one. Further effort should in future studies be invested into confirmation of the quality control candidate genes.

Single cell characterization dilemmas Approachs such as the one attempted in the present study are quite ambitious and require further testing. Indeed, great incite could be obtained into the transcripts expressed by individual cells of a larva. It is however important to bear in mind that such an methods bear importance only in a comparative approach. Care must be taken in selecting an organism with which *Platynereis* cells would be compared.

3.4 Materials and Methods

Cell dissociation of *Platynereis* at various stages Dissociation was performed as described in Chapter 2.

Ciliated cell isolation 24 and 48hpf larvae were incubated in solutions of 0.075% Pronase in SFNSW or SFCMFSW for 3-4 hours at room temperature on a shaker at low speeds in a 15mL falcon tube. Once the solution appeared blurry, the contents were centrifuged at 4000rpm and the supernatant removed. Care was taken to leave approximately 500 μl supernatant as ciliated swimming cells (if performed in SFNSW rather than SFCMFSW) could "swim" up and become aspirated and lost. Cells were resuspended in fresh SFNSW or SFCMFSW and centrifuged again. After 2-3 washing steps to ensure limited bacterial contamination (bacteria are present at the top of the supernatant), cell-containing solution was transferred into a petri dish and cells were allowed to settle. After approximately 10-15 minutes most cells either stably adhered to the bottom of the dish or sunk to the bottom. The only cells that could be observed to "move" were ciliated cells. These cells were collected using the mouth pipetting device described in Chapter 2. If dissociation reactions were carried out on large amounts of cells and the cell density on the bottom of the petri dish impeded efficient cell collection, the cell containing solution was diluted or an excess amount of SFNSW was added after allowing cells to settle at the bottom of the petri dish, the supernatant (containing the "swimming" ciliated cells) was gently decanted into a fresh petri dish.

Blastomere isolation Blastomere isolation was performed as described in Chapter 2. Briefly, adult worms maintained at 18 C were allowed to spawn in a jam cup and their eggs collected. Observation under binoculars confirmed that eggs were indeed fertilized, by evaluating the presence of egg jelly. Eggs were from then on maintained at room temperature. After 1.5 hours or when cytoplamic flow was observed in eggs, jelly was removed by gently transferring eggs onto a 100 μm mesh and pouring an excess SFNSW to remove jelly. Eggs were further washed in SFCMFSW. Eggs were kept in SFCMFSW throughout the rest of the procedure until needed. When practically all eggs were observed to segregate cytoplasm, they were transferred into either a fresh solution of 1% Sodium thioglycolate and 0,025% Pronase or alternatively ice-thawed solution that had been stored at -20Ccelsius. If the solution had been froze-thawed several times, additional Pronase was supplemented. Embryos were incubated in the described solution for 15-20 minutes at room temperature. Remaining embryos were kept on ice to prevent cell division. Frequent observation under a binocular allowed to determine when the chorion was missing in approximately 50% of embryos and two cells can clearly be distinguished in most embryos . At this stage, larvae were transferred into a fresh SFCMFSW solution using a plastic pasteur pipette and washed. This was repeated 3x in a 6 well plate in a volume of 6mL. The pipetting/washing steps help remove the chorion from some embryos. This procedure takes approximately 5 minutes time by which emrbyos should have finished cytokinesis. Using a mouth pipetting device described in Chapter 2 individual embryos which appeared "normal" based on the criteria described in Chapter 2 were selected and pipetted up and down several

times until chorion if still present was removed and individual blastomeres separated. Each blastomere was aliquoted in a separate 6-well plate well and maintained on ice. Once sufficient quantities of individual blastomeres had been isolated or once the dechorionated embryos had undergone multiple rounds of cleavage and the desired cells were no longer available, the cells that had been maintained on ice were aliquoted into PCR-tube strips in the desired volume and immediately transferred into a liquid nitrogen bath. Only cells observed to be undergoing cell division (elongation and formation of a cleavage furrow) were selected as they were judged to be healthy and "alive". The remaining embryos which had not been initially dechorionated, were then dechorionated. When processing multiple samples or when in doubt about batch quality, embryos were incubated in the dechorionation solution (1% Sodium thioglycolate and 0,025% Pronase) on ice. In this case, dechorionation took place over longer periods of time (approximately 30-35 minutes). The same criteria were applied as for embryos dechorionated at room temperature. Similarly, untimely thawed dechorionation solutions were also utilized on embryos on ice. Once sufficient quantities of cell aliquotes had been collected and aliquoted into PCR strips in liquid nitrogen, strips were transferred to $-20\,^{\circ}\mathrm{C}$ or $-80\,^{\circ}\mathrm{C}$.

Dnase I treatment assay The Kurimoto/Tang/AB protocols does not implement a DNase I step. The CellsDirect protocol contains a DNase I treatment step. Cell extracts were diluted with 1/10 DNase I buffer DNase was added. For evaluation, DNase I digestion was tested in low volumes for different amounts of time: 5, 10 and 15 minutes with diluted RNA to 20, 10 and 1pg (single cell quantities) and with individual cells aliquoted into each well incubated at 25, 37 and2 $42\,^{\circ}\mathrm{C}$. DNase I was then heat inactivated either in presence of 5mM EDTA or its absence at $75\,^{\circ}\mathrm{C}$. Finally, a 15 minute incubation at $42\,^{\circ}\mathrm{C}$ was judged sufficient and a heat inactivation at $75\,^{\circ}\mathrm{C}$. DNase I was diluted by 10x prior to addition to sample. 1μl per sample was utilized.

Amplification of singe-cell *Platynereis* RNA using CellsDirect$_{TM}$ One-Step qRT-PCR kit(Invitrogen) Amplification was carried out as recommended by the manufacturer.Transfer 10 μl of seawater containing cell(s) were transferred to a 0.2ml PCR tube. For cell lysis, tube was placed in a thermal cycler preheated to 75C and incubated for 10 minutes. Tube was chilled on ice and centrifuged to collect condensation in a table top centrifuge. 5 μl of DNase and 1.6 μl of 10X DNase I Buffer were added to each tube and mixed by pipetting and spinning down to collect contents. Tubes were incubated at 5C for 5 minutes in a thermal cycler. Tubes were spun down and 4 μl of 25mM EDTA were added to each tube on ice and mixed again. Tubes were centrifuged to collect contents. DNase I was inactivated by incubation at $70\,^{\circ}\mathrm{C}$ for 10 minutes. 8 μl of 5X RT Reaction Mix and 2 μlof RT Enzyme Mix were added and mixed. Tubes were centrifuged to collect contents and transfered to a thermal cycler preheated to $25\,^{\circ}\mathrm{C}$ and incubated for 10 minutes at $25\,^{\circ}\mathrm{C}$ and 20 minutes at $50\,^{\circ}\mathrm{C}$ for 20 minutes.Reaction was inactivated by heating at $85\,^{\circ}\mathrm{C}$ for 5 minutes. 1 μl of RNase H was added to each tube to remove template RNA and incubated at $37\,^{\circ}\mathrm{C}$ for 20 minutes. Reactions were chilled on ice and either directly used for qPCR or stored at $-20\,^{\circ}\mathrm{C}$. Using 1 μl per qPCR reaction approximately 30 qPCR reactions could be

performed with this approach.

Oocyte RNA isolation Individual non-fertilized *Platynereis* oocytes were aliquoted into individual PCR 96-well plate wells minimizing the amount of carry-over seawater. Denaturing solution (see text of Methods) were added. RNA was extracted by DNase I treatment (see DNAse I treatment assay above) and the resulting sample was directly utilized for amplification or stored at $-80\,^{\circ}\mathrm{C}$

Amplification of singe-cell *Platynereis* RNA using Chum RNA Chum RNA based amplification was performed as described by Tougan and colleagues [246].

Amplification of single-cell *Platynereis* RNA using the Whole Transcription Amplification Kit (Sigma) Amplification was performed as recommended by manufacturer and based on modifications suggested by [269]. Briefly, cell-containing-solution (approximately 10 μl) in PCR strips were thawed on ice and DNase I buffer (DEPC-treated) was added to a final concentration of 1x. Rnase inhibitor (Promega) and Superasin (Roche) were also supplemented. 0.5 μl of DNase I enzyme was added. The resulting solution was mixed thoroughly and centrifuged to collect condensate. Strips were incubated at $37\,^{\circ}\mathrm{C}$ for 10 minutes. EDTA (DEPC-treated) was added to a final concentration of 5mM. Strips were centrifuged again and incubated at $75\,^{\circ}\mathrm{C}$ for 15 minutes. When using WTA1 kit, WTA Library Synthesis Buffer (primers and dNTPs for RT) was diluted 8x in 10mM dNTPs as recommended by Roca-Gonzalez and colleagues. 2.5 μl of the diluted solution were mixed with 2.5 μlof WTA Library Synthesis Buffer and the gDNA-free nucleic acid sample. The mixture was incubated at $70\,^{\circ}\mathrm{C}$ for 5 minutes. 1 μl of WTA Library Synthesis Enzyme was added and mixed. Reverse transcription was performed using the following PCR program: $24\,^{\circ}\mathrm{C}$ for 15 minutes, $42\,^{\circ}\mathrm{C}$for 2 hours and $95\,^{\circ}\mathrm{C}$ for 5 minutes. The resulting cDNA was immediately chilled on ice and mixed with 300 μl of RNase-free H_2O, 37,5 μl WTA Amplification Master Mix, 7,5 μl 10mM dNTP mix and 12.5 U Hot Start Polymerase (Qiagen). The resulting mix was then separated into 5 PCR tubes each containing 70 μl. cDNA amplification was carried out using the following program:$94\,^{\circ}\mathrm{C}$ degrees for 3 minutes, 22x cycles of $94\,^{\circ}\mathrm{C}$ for 20 seconds and $65\,^{\circ}\mathrm{C}$ for 5 minutes. The results of the amplification were purified using Qiagen PCR Purification kit, examined by gel electrophoresis and quantity was evaluated using Qubit (Genecore) and Nanodrop.

Amplification of singe-cell *Platynereis* RNA using the Linear amplification protocol The linear amplification protocol used corresponds to the Chum RNA protocol without the addition of Chum RNA [246].

Amplification of singe-cell *Platynereis* RNA using the Kurimoto and Tang protocol Amplification was carried out as described by Kurimoto and colleagues [274] and Tang and colleagues [288].

qPCR on single cell *Platynereis* cDNA ABI PRISM 7500 Sequence Detection System (Applied Biosystems) equipped with 96-well blocks were used for qPCR measurements. Reactions were performed in 10 μl reactions using the AB SYBR mix. qPCR primers were designed with Primer3.

Table 3.13: Sequences of primers designed against ciliated cells

CLONE ID	Gene name	LEFT PRIMER	RIGHT PRIMER
48-1-02-G	Glutathione peroxidase	caaaggcactgtcctggtg	ggcacttccagtcaacccta
48-1-08-E	Cysteine rich protein	ggtgtacacacaagcggaca	cgttcctgtacattgttgatcg
48-2-01-F	Sperm associated antigen 8	ctgagcacggaaagaaaagc	gacctcttggaggagtgtagga
48-2-04-H	Mercaptopyruvate sulfurtransferase (mitochondrial?)	gggtctctcccatagtctcca	ttctgtgtgttgtcagccaaa
48-2-05-C	N/A	ggacgacaaggaactccaag	tagaacacccttcgtctcg
48-2-06-C	HSP40	gatagagtggatggtgacatgc	tgtttcttggcccctcttc
48-2-08-D	Ornithine decarboxylase	acttccgctttcatgtgtcc	ggaggacggtctcccagt
48-2-08-E	Protein with ankyrin repeats	caaagggcacgaggaagtc	tatctgctgttgctgcatcc
48-2-09-G	Heat shock binding protein 1	tgcagcagatgcaggataga	tcccatgtcatcaattcttgtg
48-2-10-B	Penicillin binding protien	aggttggaagagtccctcgt	cgagtgccctccagtgtt
48-3-08-H	Ataxin 7	tggttcaccaaagtgtcagg	aacctgatggttttgaagttgg
48-3-11-A	Histone H3	ccactgagttgctcatcagg	tcctgagcaatttctctcacc
48-3-12-B	β Tubulin	ccaccttgtcagtgcatcag	gcctcgttgtcaatgcagta
48-5-03-F	Cytochrome C Oxidase subunit II	tccttggcactcccatactc	tcctggttctacgatttcgtct
48-5-12-B	α tubulin	agatgcccagtgacaagacc	cggagaagaaagtgttgaagc

Primer name	Sequence (5' to 3')
18S rRNA forward	ATGGTTGCAAAGCTGAAACT
18S rRNA reverse	TCCCGTGTTGAGTCAAATTA
α-tubulin forward	GCCAACCAGATGGTCAA
α-tubulin reverse	GCTTGGTCTTGATGGTG
BPI forward	TTCAAGCAAGGTCCGTCCAA
BPI reverse	CCTGCTTCCAGCCATCCATC
SOCS forward	CAGATGGCCTGCAGGTACCAA
SOCS reverse:	TCGAGTCAATGCCACTGTCCA
MYC forward:	GCCCCTGCAGTTTCAGTTTCA
MYC reverse	TGGGGAGACAGGTTCGGATTT
BPI2 forward	CTTTTGCACAATGCTGGAGA
BPI2 reverse	AGCTGAGGGACAATCTTGGA
BPI2 reverse2	GCTGAGGGACAATCTTGGAC
BPI2 reverse3	ACTGCAGCTGAGGGACAATC
BPI2 forward4	GTCCAAGATTGTCCCTCAGC
BPI2 reverse4	CAGCCATCCATCTGGGTATC
s-ferritin forward	GAAGCAGGCATCAACAAACA
s-ferritin reverse	CTGGCAGAGCTACGTCATCA
s-ferritin forward2	GAGACCCAACCTCGTCAAAA
s-ferritin reverse2	GGCGTAGAGTTCCAGGTTGA
s-ferritin forward3	AGAGAGACCCAACCTCGTCA
s-ferritin reverse3	GGCGTAGAGTTCCAGGTTGA
7TM I forward	CTGTCGAGGGCGATAAAGAC
7TM I reverse	GGATTCCCCTGTTACGGATT
Arrestin C forward	AAACGCAAGGCATTCTCAA
Arrestin C reverse	GCAGCACAAGCAACAGATCA
SH2 forward	ATACCTCAGCCACCAGATCG
SH2 reverse	CCTGAGATTTCGTCACAGCA
α-actinin forward	AGCTTGGGAGAAACAGCAAA
α-actinin reverse	GGAGGTGAGAGTTGCACCAT
α-actinin3 forward2	AGCTTGGGAGAAACAGCAAA
α-actinin3 reverse2	GATCTGTGTTCCGGCTTTTC
caspase2 forward	TCTCGATTCGGTGAAGAGATT
caspase2 reverse	TTGGAAGTGCCAACTTCTCT
caspase2 forward2	AAACAATAATGGAAGCGAAACA
caspase2 reverse2	AATCTCTTCACCGAATCGAGA
dnapola2 forward	TGCCCAGTTATCATCAGCAA
dnapola2 reverse	TGGTGGTGGATGTCTCTCTG
dnapola2 forward2	TTGCCCAGTTATCATCAGCA
dnapola2 reverse2	TGGTGGTGGATGTCTCTCTG
ferritin hchain forward	GAAGCTTCAAACGAGGAACG
ferritin hchain reverse	GAGGGCTCCTCCTCTCTTGT
proteaseinh forward	GAATGCAAGCCCAGGAATAA
proteaseinh reverse	GACCGTGCCTTTGTGATTCT
defensepI forward	GGTCAGCAGTGGTTCACAGA
defensepI reverse	CTCCTGGAGAGTGCCATGTT
α-amylase forward	GTAGCCAATGGCCAACAAGT
α-amylase reverse	AGAAGGCAATTTGGTGGTTG
ribosomalprots4 forward	CAAGGGAGTACCCATGCTGT
ribosomalprots4 reverse	GTGGCAATGTTGACACGAAC
nadhubioxidor forward	GCCTCAAAGATCAGGACAGG
nadhubioxidor reverse	TGTACCAGTCTCCCCTCGAA
cub&suship3 forward	GACGGTTGGCCTTAATTCTG
cub&suship3 reverse	TCAGAACACAACTACAGAGCAGTC

Chapter 4

Cell culture

4.1 Introduction

Cell culture as it is known today was truly initiated only at the end of the XIXth century. In 1880, Arnold demonstrated that leucocytes can divide *ex vivo*. Indeed, the principles of cell culture began over 100 years ago when the German zoologist Wilhelm Roux showed that the neural plate from chicken embryos could be removed and maintained in warm saline solution for several days. Animal tissue explants were investigated since then in serum lymph or ascites fluid [317, 318].

Among the most critical animal cell culture experiment was performed in 1907 at Johns Hopkins University by Ross Harrison [319, 318]. Frog spinal cord explants were cultured in the presence of lymph, permitting the observation of neuronal fibers growing out of the explants. This experiment not only demonstrated that neuronal fibers derived from neuronal cells, but also introduced *in vitro* tissue culture as a technique. The explant was placed in a depression slide and covered with a coverslip, creating a 'hanging drop' culture, a method that had previously only been used in microbiology. He used fresh frog lymph as a culture medium and once clotted the lymph successfully sealed the culture from contamination acting as a sort of glue. This groundbreaking work provided a method that was subsequently used by many, the most notable of which was Alexis Carrel, a surgeon that was awarded the Nobel Prize in Physiology and Medicine in 1912 for his work on suturing together blood vessels whilst preventing blood clotting [320, 321, 322, 323, 318]. The aseptic techniques he used as a surgeon were decisive in his success in tissue culture, allowing him to culture and pass explants for over thirty years, eventually outliving Carrel himself, well before the advent of antibiotics. Carrel originally adapted Harrison's hanging drop method which he used to culture embryonic and adult tissues from a variety of organisms and subcultured these explants by cutting them and transferring them to fresh clots. Subsequently, a number of chambers and flasks were devised in collaboration with Charles Lindbergh, an engineer best known for his solo flight from New York to Paris in 1927 [324, 318]. Carrel was also the first to experiment with media by diluting plasma, serum and chick embryo extract with saline solutions. The pioneering methods developed by Carrel and his team

rapidly became routine and little change was introduced in the methods utilized until the 1950s, when a concerted effort was made to develop a vaccine for a devastating poliomyelitis epidemic. There were two main challenges in this regard. Cells grown in explants did not provide a large enough yield for vaccine production. A vaccine containing cell culture media consisting of embryo extract or animal sera would risk of provoking an immune reaction in patient treated. The first problem was resolved with a method developed by George Gey, consisting of a 'roller tube', which allowed cells to migrate and form a monolayer on the sides of tubes [325, 318]. This method was adapted by John Enders to produce high titer vaccinia virus and Robbins and Weller grew mumps and varicella virus in suspended human fetal cultures [326, 318]. The rediscovery of trypsin which went unnoticed in 1916 when Rous and Jones published a report on its benefits, in 1950 revolutionized tissue dissociation. Thanks to this, the virus titer could be determined, allowing Jonas Salk to determine that three different strains of virus had to be included in the vaccine.

Fischer and White were among the first to development synthetic media rather than using fluids. Their pioneering studies were taken up subsequently by Eagle on HeLa and mouse cells [327, 328, 329, 330, 331, 332, 333] Concomitantly, phase contrast and inverted light microscopes became commercially available [318]. Earle was among the first to succeed at single cell cloning, albeit at a very low success rate. The second problem was thus resolved when the first artificial medium lacking serum designed for the culture of chicken embryo tissue by Morgan Morton and Parker was used used by Arthur Franklin to grow polio virus in monkey kidney cells [334]. In 1954 Enders, Weller and Robbins received the Nobel Prize for Medecine for their cell culture work to elaborate the polio vaccine. Soon after, protein free media became available. By careful titration, Han's F-12 medium was developed [335].

The exciting advances observed in vertebrate cell culture could not go unnoticed by the invertebrate cell culture field. Insect cell culture particularly benefited from the advances in vertebrate research. Vertebrate protocols were applied to arthropod cells. At first attempts were made to replicate Carrel's efforts in vertebrate tissue growth of explants in "plasmatic media" in hanging drop culture. Mitosis was however only rarely observed. For this reason, effort was put into adapting the existing culture media empirically by modifying vertebrate-based media composition. Insect-derived tissues survived for some days or sometimes weeks with no significant cell growth until the early 1930ies. William Trager used information about insect hemolymph composition and devised his own medium, with the purpose of studying virus replication *in vitro* in silkworm ovaries and observed mitosis. The most vital advances in the field did not take place until the 1950ies and 60ies, when antibiotics became commercially available. Silva Wyatt analyzed insect hemolymph to design a further medium which has been the basis of multiple media used subsequently [336]. She observed mitosis in cells that survived for over 3 weeks. Wyatt's study was exceptional in that she had carried out rigorous studies on the biochemistry of body fluids, which allowed her to develop a complete medium. Her pioneering work paved the way for Thomas D. C. Grace who using information from vertebrate media added vitamins and Krebs cycle components, through a technique he described as "organized neglect". Grace developed the very first continuous invertebrate cell line from *Antheraea eucalypti*, Australian Emperor gum moth ovaries [337].Schneider similarly devised a synthetic medium for *Drosophila* cell

cultures, also based hemolymph analysis.

The fervor exhibited at developing carefully titrated and fine-tuned media corresponding to physiological solutions was paralleled in stark contrast by attempts to a simple minimal medium. Mitsuhashi and Maramorosch for example designed a medium for leafhopper cells consisting of salts, glucose, lactalbumin yeastolate, fetal bovine serum (FBS) and antibiotics [338, 339, 340, 337], whereas Mitsuhashi developed a medium consisting of seawater, yeastolate, lactalbumin hydrolysate, and table sugar, which supported fifteen different cell lines[337]. This kind of approach had several advantages, practicality of manipulation set aside. On one hand, unlike vertebrate sera that were commercially available in the 1960ies, invertebrate hemolymph was difficult to obtain in large volumes. Substituting it with simple ”bulk” mixtures made cell culture cheaper and more accessible. Numerous media have since been developed based on either approach - the rigorous biochemical examination of body fluids or the minimal medium consisting of mixtures of solutions of unknown composition. Most of these media are designed to suit vertebrate and terrestrial insect cells. This is striking as a considerable portion of the diversity of life on Earth does not so to speak ”have access” to defined cell culture conditions. This is not surprising.

Practically speaking, polio research re-energized cell culture in the 1950s establishing methods to culture, feed, subculture, manage contamination and measure culture changes of cells. This research did not arise on its own. In the United States where a considerable portion of the effort was taking place benefited from the constant support of organizations such as the March of Dimes founded by US President Franklin D. Roosevelt, himself a famous victim of polio. Without the support and effort put into finding a polio vaccine, vertebrate cell culture may have taken considerably longer to develop, as we can see with marine invertebrate cell culture. Indeed, despite numerous local organized efforts to develop long-term cell cultures by academic and commercial groups working on marine invertebrates with serious economic incentives [341], to date there exist no marine invertebrate cell lines [342, 343].

This is not to say that there have been no cell cultures of marine invertebrate in the past sixty years. Marine organisms have been studied, dissociated and placed in cell culture media for decades. However, lack of information about their general respective physiology and biochemistry, has hindered the production of any lineage-specific cell culture media. Cells can therefore be routinely grown in primary culture, but cell lines have not yet been obtained successfully[342, 343].

4.2 Results

4.2.1 Evaluation of salinity tolerance and salt requirements of *Platynereis dumerilii* cells

The phospholipid bilayer surrounding cells is relatively impermeable to ions and other solutes, but somewhat permeable to water. Permeability to water can be increased by the presence of water-channels. Water moves across membranes from a solution of low solute concentration to one of high, in a process called an osmotic flow. Different organisms cope with osmotic pressure by different methods. *Platynereis dumerilii* is

a marine organism, widely distributed in European intertidal waters [344]. Saltwater species generally are in near osmotic equilibrium with seawater and must necessarily regulate intracellular composition. Brackish water species and those saltwater species that migrate into estuarine habitats may regulate or resist osmotic challenge but in most cases are capable of osmotolerance. This is the category *Platynereis* falls in to. *Nereis virens*, a close relative of *Platynereis* is known to have a medium osmotic pressure of 1000 mOsm/L and coelomic fluid osmotic pressure of 1048 mOsm/L which is comparable to that of seawater. Solute uptake has been shown to take place through the skin and solute expulsion in adult organisms takes place through nephridia.

As many marine polychaetes, *Platynereis* larvae and adults are iso-osmotic. It has been suggested that osmo-conforming annelids may be dependent on cellular mechanisms of osmoregulation. Cells exposed to moderately hypotonic media swell by osmotic water uptake, and then shrink again in a process known as the regulatory volume decrease, via solute release from cells through specific membrane channels. P-type ATPase has been uncovered in *Platynereis dumerilii*. Adult nereids have been shown to regulate their osmotic pressure through uptake and release of amino acids [345].

No study of the osmotic tolerance of *Platynereis* cells *ex vivo* was found in literature. In order to determine the range of salinity tolerated by *Platynereis* cells, different concentrations were tested by diluting seawater in a standard cell culture medium (DMEM). The osmolarity of DMEM is 300mOsm/L. The osmolarity of seawater is 1100 mOsm/L. Cells derived from larvae and adults were tested in this range. The results are summarized in Table 4.2.1).

Table 4.1: Summary of evaluation of salinity tolerance

Mix used	Osmolarity mOsm/kg	Effect on larval cells	Effect on adult cells
DMEM alone	300	Died within 20 min	Died within 20 min, Large cells survived
DMEM : SFNSW 75%:25%	500	Died within 20 min	Died within 20 min, Large cells survived
DMEM : SFNSW 50%:50%	700	Most died within 20 min some survived	Died within 20 min, Large cells survived
DMEM : SFNSW 35%:65%	820	Some died within 20 min most survived	Some small cells died, large cells survived
DMEM : SFNSW 25%:75%	900	Most cells survived	Most survived
SFNSW alone	1100	Most cells survived	Most cells survived

Cells derived from adults and larvae were observed to swell and then leak out cytoplasmic content within 20 minutes at low osmolarities (300 mOsm/kg -700 mOsm/kg), experiencing what was assumed as an osmotic shock. This effect was also observed in 24hpf-old larvae. Large cells derived from adults were less prone to osmotic shock. Cells that morphologically appeared to be muscle-like or coelomocyte-like did not leak cytoplasm or change morphology for 24 hours in culture. At osmolarities ranging from 820mOsm/L to 1100mOsm/kg most cells and larvae survived and less cell death was

observed as compared to lower osmolarities (Table 4.2.1).

Based on these results it was concluded that in all subsequent attempts to culture *Platynereis* cells, osmolarity needed to be adjusted to 820 mOsm/kg to 1100mOsm/kg, for the survival of the largest amount of cells. The reasons behind osmotic tolerance by large adult-derived cells were not investigated further. Muscle cells are known for their intrinsic capacity for the tight control of ionic fluxes, this may make them immune to changes in salinity in the environment. Coelomocytes include elaeocyte (macrophage-like cells responsible for phagocytosis of muscle tissue during metamorphosis). They are characterized by the presence of large acidic vacuoles. They therefore also have an intrinsic capacity to control ionic concentrations.

4.2.2 Determination of the pH requirements of *Platynereis dumerilii* cells

The pH of tolerance of organisms can vary significantly and is closely linked to their capacity to regulate ionic flow and osmotic pressure. *Platynereis dumerilii* early embryos, young larvae and dissociated cells were exposed to iso-osmotc NaCl solutions (33g/L) with pH adjusted to 1, 3, 5, 6, 7, 9 and 10.

Table 4.2: Summary of qualitative evaluation of pH tolerance (see text for details)

pH	Dissociated cells	Embryos	Trochophore larvae
1	-	-	-
3	-	-	-
5	-	+	+
6	+	++	+
7	++	++	++
9	+++	+++	+++
10	-	-	+

Cells and larvae died rapidly at pH lower than 3. Cells were observed to change shape or lyze rapidly. Embryos and larvae changed color to slightly yellowish and eventually brown color within 15-20 minutes. Older trochophore larvae (over 30hpf) ceased swimming and sunk to the bottom of the 6 well plate, prior to color change. However, at pH 5 cells, embryo and larvae were observed to survive. Early cleaving embryos did not die immediately but ceased cleavage prior to 12hpf stage. No trochophore development was observed. At pH 6 to 9, no change in color was observed. Embryos and cells continued to cleave throughout the period of observation (3 hours). Trochophore larvae appeared similarly to not be affected in a deleterious fashion at pH 6, 7 and 9. No morphological difference could be observed between these pH values for larvae, embryos and cells. At pH 10 some cells were observed to change shape and die. Larvae and embryos became more transparent, with a slight blue tint. Invariably cleavage was delayed eventually resulting in larval and embryonic death. Moreover, it was frequently observed that dechorionated embryos at slightly acidic pH underwent even cleavage as opposed to asymmetric cleavage during the first cleavage stages. Although quantitative evaluation was not carried out, a change in pH of dissociation

solution clearly affected cleavage pattern, resulting in an increase of misdeveloppers as depicted in Fig. 2.12.

The best conditions for rearing *Platynereis* embryos and larvae appeared to be indeed slightly alkali pH (7-9). This is inline with observations performed by Batten and Bamber on *Nereis virens* adults [346]. The latter described an increase in mortality, burrowing activity and dry weight as well as decreased glycogen levels in survivors over a period of 10 days in seawater at pH 6 and lower, indicating stress. In the present evaluation, tests were not carried out on adults *Platynereis* due to shortage of worms. A trend in terms of higher resistance to pH was indeed observed between young and old larvae, with older larvae showing higher resistance to acidity.

In respect to cell culture, it is important to bear in mind that most vertebrate and insect cell and tissue culture is carried out at neutral pH, indicating the necessity of culture medium buffering to acidic environments. Embryos and larvae were more resistant to low pH than dissociated cells. The chorion may be providing protection against harsh medium conditions such as acidity and alkali. The permeability o f

4.2.3 Determination of antibiotic and antifungal requirements and tolerance of *Platynereis dumerilii* cells

Microbiological contamination of cells cultured *in vitro* has been a predominant problem since the very beginnings of the field. However, using only aseptic techniques cell lines derived from vertebrates were indeed cultivated. As demonstrated by Alexis Carrel, cells could be cultured long before the advent of antibiotics using complex sterile techniques, however these stringent techniques were a serious deterrent to scientists wishing to join this field and were alleviated by the commercial availability of antibiotics [347]. This problem is particularly pertinent in regard to cell culture initiated from marine invertebrates as starting material used is rarely reared in septic conditions. Such contaminations with protozoa, bacteria and fungi are a major cause of loss of most marine invertebrate cell cultures [125, 347]. Surprisingly, in contrast to media component composition which have been standardized for decades and are commercially available, antibiotic concentrations are not standardized at all [347]. Indeed, Kuhlman surveyed 678 publications from three scientific journals concerned with cell culture [347] and observed a wide range of concentrations used. Penicillin and gentamycin were present from 1% to 10% of medium, streptomycin from 0.5μg/ ml to 10 mg/ml of medium, Amphotericin B or fungizone from 0.25 to 250μg/ml, Nystatin up to 100 μg/ml, and kanamycin and neomycin up to 60μg/ml. Moreover, several antibiotics are often used simultaneously in a further lack of consensus. Of the 243 vertebrate cell culture studies in which antibiotics were added to the medium, Kuhlman remarked 85 different reported combinations [347]. The lack of standardization not only impedes on comparisons between experimental setups, but may also affect the outcomes of cell culture experiments. Indeed, the effect of antibiotics depends on the composition of the media, namely the protein content and pH. The optimal pH for most antibiotics is between 7.5-8.0, which is in contrast to the optimal pH of *Platynereis* larvae and cells (see above). The main protein component routinely added to cell culture is of course serum. However, not only has the presence of serum albumin been shown to affect penicillin

activity [348], but different species of serum demonstrate different binding affinity to penicillin [349]. The most commonly used antibiotics in cell culture can be subdivided into four large classes based on their mode of function: inhibition of bacterial cell wall synthesis, inhibition of cell membrane synthesis, inhibition of protein synthesis and inhibition of DNA synthesis. β-lactam antibiotics such penicillin and its derivatives affect cell wall synthesis in bacteria. Polyene makrolides such as amphoptericin, fungizone and nystatin inhibit cell membrane formation in fungi. Aminoglycosides (streptomycin, neomycin, kanamycin or gentamycin), markolide (tylosin) and tiamulin all function via different pathways to inhibit protein synthesis. Quinolone-based compounds such as ciproflaxin inhibit DNA synthesis. These different classes of antibiotics and antifungals act via different methods and *a priori* should only affect target contaminants. However, mounting evidence clearly demonstrates that this is not the case. There are conflicting reports concerning polyene macrloide antimycotics such as amphotericin B, fungizone and nystatin which have been on one hand claimed to not affect the functioning of non-fungal cells and on the other hand to affect permeability of muscle cells [350]. β-lactam antibiotics such as penicillin interact with cell wall proteins present in bacterial cells, that are in principle absent in eukaryotic cells and should therefore result in low toxicity if any at all. Lange and Assmann however demonstrated that serum albumin protein could bind to penicillin and could hypothetically facilitate entry into the cell. [351]. Once in the cell, the β-lactam antibiotics have been proposed to bind DNA polymerase α, resulting in a cell cycle dependent effect on DNA synthesis [352]. Aminoglycosides have been observed to affect proliferation and protein synthesis in a variety of vertebrate cells, with particularly strong toxic effect observed on renal cells. Gentamycin was shown to accumulate in lysozomes [347]. Based on mounting evidence that prophylactic supplementation of cell culture with antibiotics results in numerous unaccounted repercussions on the cells, Kuhlman advised against prophylactic supplementation all together and argued for therapeutic supplementation only and the need for a developing consensus standard concentrations specific to each cell line [347]. If one bears in mind that these observations were carried out on vertebrate (primarily mammalian) cells, it becomes evident that the effects exerted by these substances on novel model organisms such as *Platynereis* is difficult to extrapolate. For this purpose it was essential to establish the optimal antibiotic and antifungal supplementation concentrations. *Platynereis dumerilii* resistance to antibiotics has not been characterized. Therefore, different concentrations of a select group of frequently used antibiotics were tested at recommended concentrations, in "minimal media" containing serum (sea below):

Cell membrane inhibitors :	Nystatin, Amphoptericin B
Protein synthesis inhibitors:	Kanamycin and Streptomycin
β-lactam antibiotic:	Penicillin
Antifungal and antibiotic:	$CuSO_4$ and Unimarine

A quantitative and qualitative examination of the effects of these substances was performed using the MTT-reduction assay. Nystatin precipitated in seawater and cells incubated in copper sulfate invariably succumbed to bacterial contamination, rendering a quantitative evaluation impossible and demonstrating their inefficiency at preventing

contamination.

Previously, *Nereis virens* oocytes have been cultured in presence of 100U/ml penicillin and 129 μg/ml dihydrostreptomycin sulfate with 100 μ g/ml gentamicin[163]. *Nereis virens* eggs have also been used as biocarriers for the delivery of oxytetracycline antibiotic to predator larvae loaded with concentrations reaching 1μ g per egg[353], indicating a possibly high tolerance of antibiotic compounds by nereids. Penicillin and Streptomycin, supplied as a single solution (GIBCO) were tested together. Quantitative evaluation of the effect of Penicillin and Streptomycin demonstrated that both larval-derived and adult-derived cells (Fig. 4.2.3top panel) displayed higher viability in presence of 10U/ml Penicillin 0,01mg/ml Streptomycin and that larva-derived cells additionally survived successfully in presence of 100U/ml Pencillin 0,1mg/ml Streptomycin(Fig. 4.2.3top panel). This is in line with the range of concentrations utilized in vertebrate culture.

Incubation in presence of Kanamycin (Fig. 4.2.3:bottom right panel) was tested on both larval and adult cells and proved deleterious to adult cells. Larval cells systematically succumbed to bacterial contamination, indicating a lack of efficiency of kanamycin to combat contamination and impeding any quantitative evaluation of its effect of cell metabolism and survival. Adult cells incubated in presence of the lowest concentration tested (20μ g/ml) had the highest viability, indicating a strong toxic effect on *Platynereis* cells. Kanamycin is commonly used at concentrations two-fold or higher in vertebrate cell culture. Thus, it can be hypothesized that *Platynereis* cells may be more sensitive to the action of kanamycin than vertebrate cells. The mode of action of kanamycin is similar to that of streptomycin (protein synthesis inhibition by an aminoglycoside), however, streptomycin in conjunction with penicillin did not affect *Platynereis* cells as severely. From the experiments performed, it is impossible to conclude whether there is a difference in the sensitivity of *Platynereis* cells to kanamycin and streptomycin, as streptomycin would have to be tested alone and compared to the streptomycin and penicillin cocktail. Steptomycin alone was not available at the time at which experiments were carried out in the lab. For the needs of the present pilot study, this experiment was judged to be unnecessary. Based on the present results, it was concluded that kanamycin was deleterious to *Platynereis* cell culture and Steptomycin and Pencillin were not, as judged from metabolic quantification using the MTT-reduction assay.

Both larva-derived and adult-derived cells incubated in presence of 200μ g/ml Amphoptericin B survived best(Fig. 4.2.3:bottom left panel). Additionally, larva-derived cells survived in 100μ g/ml Amphoptericin B. The difference in sensitivity between larval and adult cells was not investigated further. These results are in line with the range of concentrations used in vertebrate cell culture.

As *Platynereis* are fed with a variety of algae and several microorganisms are present in their breeding environment. Adult *Platynereis* contain these organisms in their gut and therefore any culture based on such adults will invariably contain protozoa and algae. Fertilization is external and consists of adult organisms emptying the contents of their abdomen (primarily gametes) but also the other contents in to the seawater. Thus embryos obtained from this kind of culture will also contain some algae and marine eukaryotic microorganisms. For this reason, establishing a method to prevent these unicellular eukaryotic organisms from proliferating was essential. Uni-

marin Aquarium Munster is a universal treatment against the numerous infections of marine ornamental fish such as protozoa (e. g. *Cryptobia, Cryptocarion*), bacteria (e. g. *Flexibacter*) and fungus (e. g. *Saprolegnia*). 1 ml of Unimarin contains 4 mg ethacridine lactate, 2.75 mg tetramethylthionine chloride, 2 mg acriflavin chloride, 0.26 mg 4-(4'-dimethylaminophenylazo-benzol-1-sulfonic acid) sodium salt. Although the manufacturer indicates that invertebrates do not tolerate it and that corals and anemones must be excluded from treatment, *Platynereis* larvae and cell treatment was attempted with minimal doses. Larvae do not appear to be negatively affected when breeding in seawater supplemented with Unimarin at recommended dosages (one drop per liter of medium). Preliminary observations indicated higher survival of larvae derived from oocytes fertilized and maintained in presence of Unimarin for 24 hours. The uptake of Unimarin by *Platynereis* cells could be observed at concentrations from 1μl/L and higher. Incubation at lower concentrations were not attempted. Large green crystalline precipitates were observed. Not all *Platynereis* cells displayed the capacity to take up the Unimarin. In some cases the whole cell was stained green whereas in other cases only a small circular structures displayed green d ye. Not all cells took up the Unimarin, many cells were observed to not contain green dye within the same aggregate and in separate aggregates. The cells which contained the green dye did not co-locolize with Trypan blue, indicating that the dye was not immediately toxic to the cells (did not precipitate cell death) nor was it not uptaken only by dead cells. Furthermore, cells stained green with Unimarin remained in the cell culture dish for up to 2 weeks, indicating a lack of toxicity. It was concluded that certain *Platynereis* cell display the capacity to take up Unimarin or the dye present within from their environment whilst others cannot. Upon incubation for periods extending over a week in Unimarin, absence of cell division was observed as compared to samples not containing Unimarin. The difference between cells capable of taking up green coloring and those incapable of doing so was not established. The vacuolar structures containing the green dye are similar to the coloring observed upon Neutral red live staining. Neutral red is s a eurhodin dye that stains lysosomes red. The uptake of neutral red depends on the cell's capacity to maintain pH gradients, through the production of ATP. At physiological pH, the dye presents a net charge close to zero, enabling it to penetrate the membranes of the cell. Inside the lysosomes, there is a proton gradient to maintain a pH lower than that of the cytoplasm. Thus, the dye becomes charged and is retained inside the lysosomes [354]. If the structures stained by Unimarin are the same ones as those stained by Neutral red, this would imply that uptake of Unimarin is an active process and that it only takes place in living cells. As the Unimarine is distributed as a dark green solution, colorimetric tests cannot be performed.

Based on this preliminary evaluation, it was concluded that Kanamycin and Nystatin were not suitable for *Platynereis* cell and tissue culture, whereas pencillin, streptomycin, amphoptericin B, nystatin and Unimarin were. The most appropriate concentrations of each compound tested (10U/ml Penicillin, 0.01mg/ml Steptomycin, 200 μg/ml Amphoptericin B and 1 μl/L Unimarin were used in all subsequent experiments unless indicated otherwise.

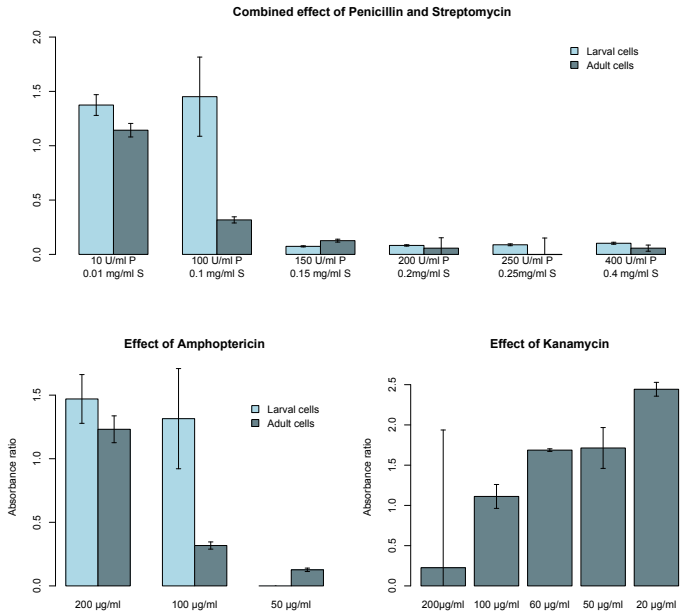


Figure 4.1: Effect of antibiotic and antifungal substances on *Platynereis* adult and larva-derived cells. Top panel: The combined effect of Penicillin and Streptomycin on larval and adult cells as determined by using the MTT-reduction assay. Best viability was observed at the lowest concentrations tested. Bottom left panel: Effect of Amphoptericin B on larval and adult cells as determined by the MTT-reduction assay. The best viability was observed at the highest concentrations tested (200 microμg/μl). Bottom right panel: The effect of Kanamycin on Adult cells as determined by the MTT-reduction assay. The best viability was observed at the lowest concentrations tested (Larval cells: light blue, Adult-derived cells: dark blue).

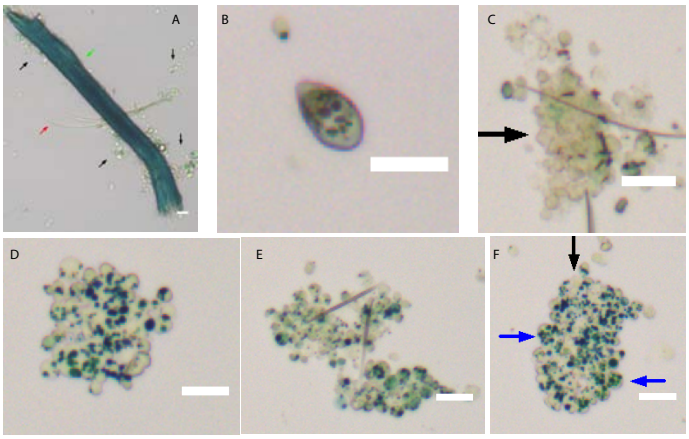


Figure 4.2: Treatment of *Platynereis* cells with Unimarine (A) Large green precipitates were observed in minimal medium supplemented with 1μg/L Unimarine (B) Protozoan contaminant displaying green coloration indicating uptake of Unimarine (C) *Platynereis* cell aggregate in which few cells took up Unimarine (black arrow) (D) *Platynereis* cell aggregate in which cells took up Unimarine in a non-uniform manner staining only a subcellular component (E) *Platynereis* cell aggregate in which some cells were entirely staining with Unimarine and other cells were only partially stained by Unimarine (F) *Platynereis* cell aggregate in which numerous cells took up Unimarine green coloration (blue arrows) and some cells did not take up Unimarine (black arrow) Scale bar: 30μm

4.2.4 Determination of the serum supplementation requirements and tolerance of *Platynereis* cells

Cell proliferation is dependent on numerous factors such as the presence of growth factors. Among the most common growth factor supplements is the hemolymph or serum of the organism from which the cells are derived (see Introduction). Due to its commercial availability Foetal Calf Serum (FCS) is also commonly used in cell and tissue culture(see Introduction). Although the composition of serum is not clear, some components are known as albumins, immunoglobulins, assorted proteins, chemokines, growth factors, minerals, vitamins and some complex lipids. The main component of serum of mammals is albumin. *Platynereis dumerilii* larvae are free-swimming and no foetal serum can be obtained. Adult *Platynereis dumerilii* are small (rarely exceeding several centimeters in length) and are therefore not easily amenable to large scale hemolymph extraction. *Nereis virens* is close relative of *Platynereis dumerilii* which can reach up to 1 meter in length. Therefore, hemolymph and coelomic fluid was procured from maturing *Nereis virens*. Supplementation of seawater with *Nereis virens* hemolymph was compared with supplementation with *Nereis virens* coelomic fluid, with FCS and with bovine albumin. Cell viability after 24 and 48 hours was determined using the MTT-reduction assay.

Invariably high concentrations of FCS yielded the highest cellular viability (Fig. 4.2.4), although variability between different batches of serum were observed. This effect was not due to the presence of the main component of FCS, albumin incubation with which alone had a deleterious effect on cellular viability (Fig. 4.2.4). Cellular viability in presence of NVH was consistently lower as compared to in presence of FCS (Fig. 4.2.4), as demonstrated both by an MTT-reduction assay and an FDA assay (Fig. 4.2.4). An increase in cellular viability was observed over time when measurements were performed for a period of 48hours (Fig. 4.2.4). Putative components of FCS, EGF, BMP4 and an Insulin-based solution could not account for the effect produced by FCS individually, resulting in low cellular viability (Fig. 4.2.4). Alternative serum source chick egg extract resulted in comparable effects at 10% however a significant decrease in cellular viability was observed at the highest concentration tested (20%). As numerous growth factors act synergistically, the effects of EGF, BMP4 and insulin were further investigated. Personal communication from Dr. Nelly Odintsova (Institute of Marine Biology FEB RAS, Vladivostok, Russia) highlighted the need for fetal fluid for cell culture rather than adult hemolymph.

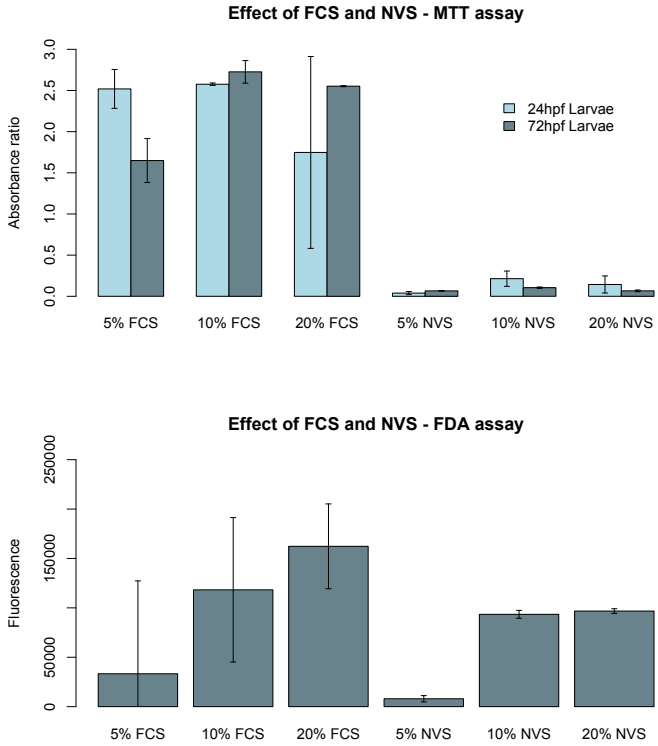


Figure 4.3: Effect of different sera on cell viability estimated using the MTT-reduction assay and the FDA assay. Effect of different concentrations of FCS and *Nereis virens* hemolymph after 24 hours in culture and 48 hours (top panel) and using FDA after 24 hours(bottom panel).

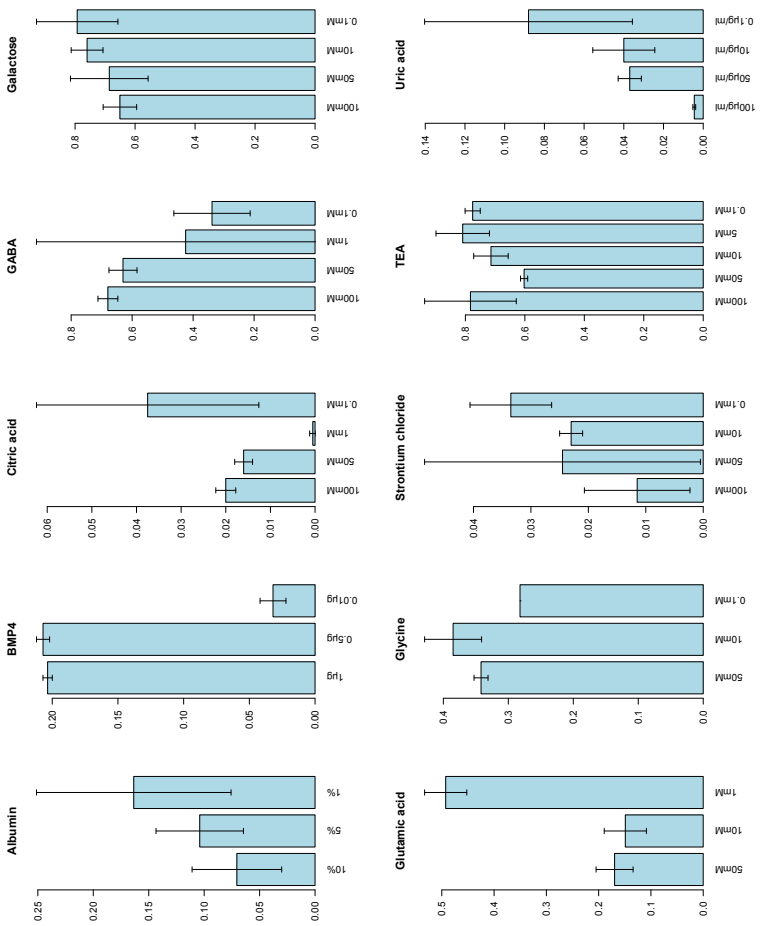


Figure 4.4: Effect of various additives on cell viability estimated using the MTT-reduction assay at different concentrations.

Attempts were made to reconstitute *Platynereis* nutrient composition empirically by addition of fluid derived from heat treated and macerated larvae and adults. This attempt failed as heat inactivation (at 65 °C or 75 °C for 15 minutes) was unsuccessful at neutralizing bacterial and protozoan contaminants. Cultures treated with all concentrations of this mix consistently succumbed to contamination, rendering a quantitative and qualitative evaluation impossible. Attempts were also carried out using lyophilized *Nereis virens* extract. Although, the lyophilized extract was resuspended in sterile and filtered water, contamination was observed with both bacteria and fungus, impeding further characterization. Macerated non-fertilized eggs were also tested. A single batch corresponding to approximately 1500 non-fertilized eggs were obtained from a mature *Platynereis* female by dissection her abdomen open and washing the contents over a 100 μm nylon mesh. Eggs were frozen in liquid nitrogen and subsequently thawed and heated to 65 °C for 20 minutes in a total volume of 0.5 ul. Cells incubated in identical conditions as those utilized for the MTT-reduction assay described above survived for 5 days before succumbing to contamination. In this preliminary experiment, quantitative evaluation was not attempted. Although, this observation of *Platynereis* cells in the presence of oocyte extract was quite promising, this evaluation was not continued further due to the limitation of starting material. For each cell culture experiment several batches of non-fertilized eggs would need to be sacrificed in parallel. This was deemed infeasible, at the time when these experiments were carried out.

4.2.5 Determination of Minimal Media requirements and tolerance of *Platynereis* cells

As described in the Introduction to this chapter, another obstacle in invertebrate cell culture that has been brought up by numerous authors is the lack of data on the *in vitro* physiology of the cells and the resulting absence of appropriate culture media and conditions. This has been the main argument explaining the low frequency of mitosis [355, 125]). Few extensive and systematic studies of media components and culture conditions have been undertaken in selected organisms[125, 356] . A few attempts have been made based on natural and artificial seawater [357, 358]. Nevertheless, most culture media reported to date consist of balanced saline solutions complemented with carbohydrates and amino-acids and antibiotics. One strategy as described above is to mimic the composition of hemolymph of the source organisms that is put into culture [359](see Introduction). The second strategy is is to derive culture conditions from commercial media and modify it by addition of salt solutions to adapt osmolarity to an acceptable range for marine organisms(see Introduction to this chapter).

The first approach was attempted by using a simplified "minimal medium" consisting of seawater supplemented with MEM vitamins, 10% FCS and antibiotics, which will be referred from now on as Minimal medium1 (MM1) grown in Nunc 24-well plates (Fig.4.5 A-C; Fig 4.6A-H).

Cells derived from 6hpf larvae incompletely dissociated larvae were placed, cultured and observed at regular intervals. After 6hours in culture, cell aggregates clustered together (Fig. 4.5 A) These aggregates did not adhere to surface of culture vessel. Small transparent round cells were observed outside the aggregates. These cells ad-

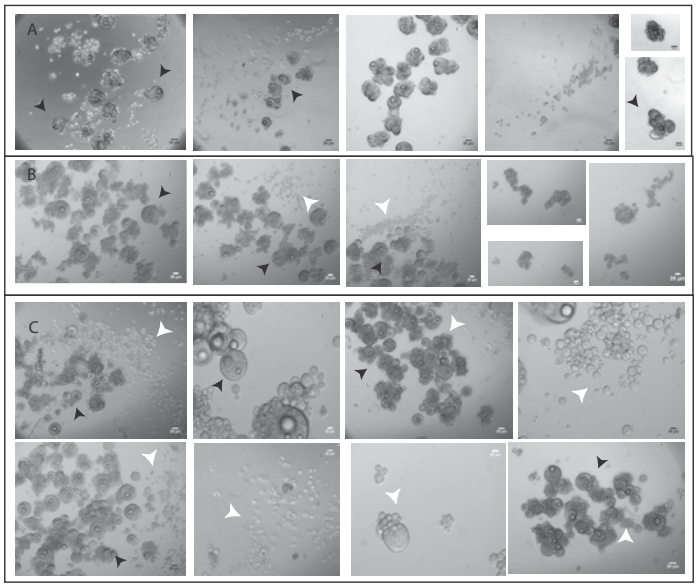


Figure 4.5: Effect of Minimal medium of embryonic primary cell culture. Cells derived from 6hpf larvae from incomplete dissociation, grown in 24 well plate in MM1 medium. After 6 hours in culture: cell aggregates (black arrowheads) formed larger aggregates but did not adhere to plate surface, whereas individual cells (white arrowheads) adhered to plate surface (A). After 2 days in culture (B): larger quantities of individual cells were observed as compared to 6 hours in culture, many cells not belonging to a defined cell clusters appeared larger than the cells observed at 6hpf . After 3 days in culture (C): number of cells not clearly belonging to any defined cell clusters increased, more small cells were observed forming further cell loosely bound aggregates of cells between the previously observed aggregates

hered to plastic as individual cells or formed aggregates. Adhering aggregates were not observed. The propensity to adhere to other cells and other aggregates appeared higher than that to adhere to plastic. Formation of large clusters of aggregates was observed (Fig. 4.5 A). It could not be concluded whether a diffusible signal or cue was produced by cells.

After two days in culture, (Fig.4.5 B) incompletely dissociated aggregates remained. Aggregates did not adhere to surface of culture vessel. Numerous cells smaller in size than those within the aggregates were observed. These cells either attached to the aggregates or segregated from them in ”waves” of cells (Fig.4.5 B).

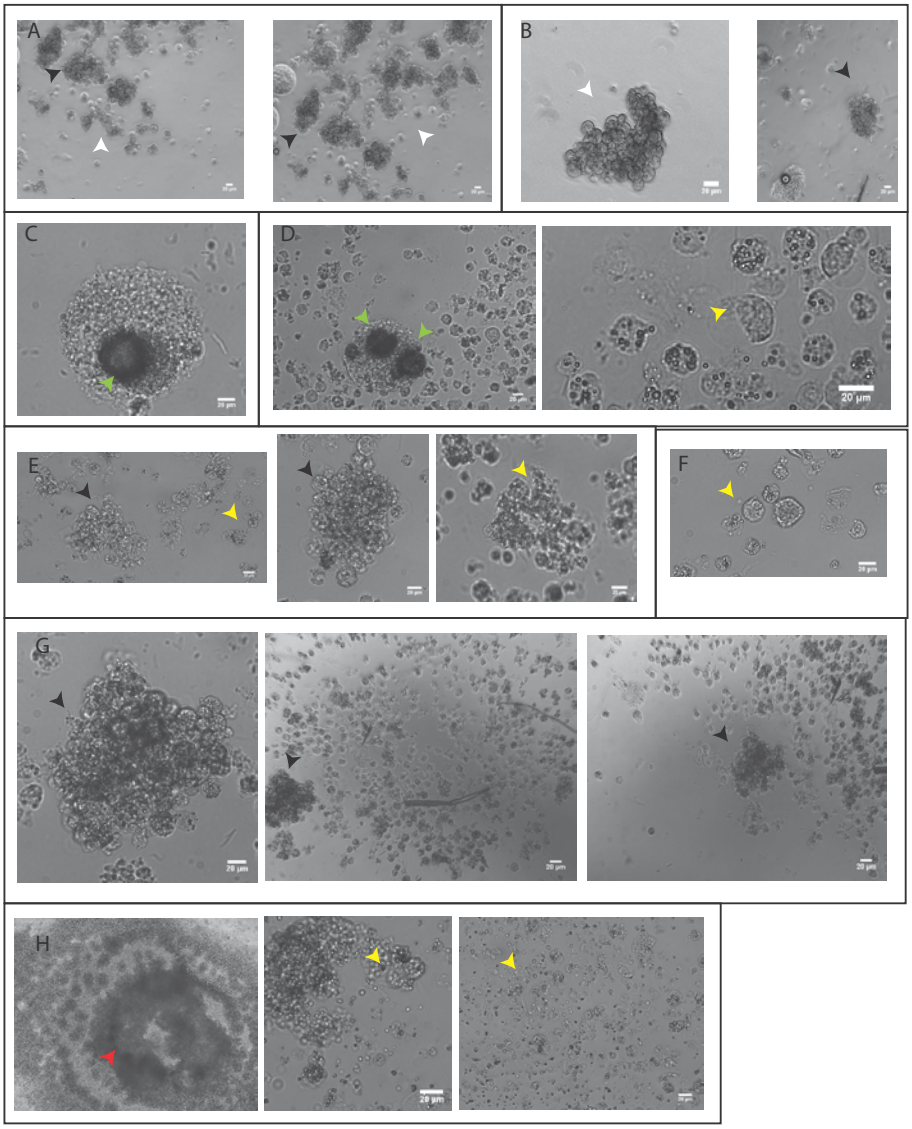
After 3 days in culture, aggregates were still present and did not adhere. Cells outside of the aggregates appeared larger in size and adhered to plastic. Adhering cells formed clusters. More smaller cells were observed between the aggregates. These cells did adhere mildly to plastic. Light shaking unattached these cells easily (Fig. 4.5). Due

to the ease in disaggregating the aggregates attempts at counting the number of cells were not conclusive. Overall more smaller cells were observed overtime. Whether the cells "shrank" or were the result of cell division was not established. Cells were incubated for 12 hours in culture and were sacrificed after 3 days in culture. Unfortunately, most cells were lost during the washing steps in the staining protocol, and no quantitative conclusions could be made about the capacity of these cells to proliferate *in vitro* in the presence of Minimal Medium 1. Subsequent attempts to replicate this culture were carried out in Nunc 6-well plates. Cells did not attach to the substrate as well as in 24-well plates. Most cells observed remained in suspension or settled to the bottom of the dish without attaching to its surface in a stable fashion. Cells could be kept in culture for up to 29 days with a change in media every 3 days. This experiment was carried out in several replicates (n= 18 wells of 5 ml media). Of the 18 wells, 9 succumbed to contamination within the first week of culture. The contaminated cultures were located in different plates and in the course of the second week. Contaminated wells were drained and treated with ethanol. However, within the course of the following week, all cells located in the plates that had shown signs of contamination were contaminated with fungi and had to be sacrificed. The cells and cell aggregates located on the remaining plate survived between (n=2) 21 days, (n=3) 26 days and (n=1) 29 days. Few signs of proliferation were observed. Both small and large cells remained present. Cell size did not vary between the sizes observed in the first week and the third week. More smaller cells were observed to be dispersed throughout the wells. After day 16 in culture cells were observed to partly adhere to the the surface of the wells. No signs of differentiation were observed. Cells remained circular in morphology. Neither pigmentation or projections could be identified. For this reason, Minimal Medium 1 was considered sufficient for rearing and maintaining cells in culture. However, the purpose of a cell culture is of course to obtain cell proliferation. For this purpose additional ingredients were deemed necessary.

Cell proliferation is the increase in the number of cells as a result of cell growth

---

Figure 4.6 *(following page)*: Effect of Minimal medium of embryonic primary cell culture with supplements. Cells derived from 6hpf larvae from incomplete dissociation, grown in 24 well plate in MM1 medium with supplements. After 2 days incubation in presence of BMP4 (5μg)(A)aggregates with numerous cells (black arrowheads)as well as individual cells (white arrowheads)were observed. The number of cells in these aggregates appeared larger those that were not treated with BMP4 and remained in MM1 (B and in an independent experiment Fig.4.5). Cells treated with BMP4 after 23 days in culture (C) demonstrating dense cell structures on the surface of aggregates (green arrowheads)and cell death (yellow arrowheads). Cells incubated in EGF 5% for 18 days (23 days total incubation time)(D) displaying similar dense structures on the surface of aggregates (D-left, green arrowheads) and extensive cell death (D-right, yellow arrowheads). Cells incubated for 18 days in presence of 5% EGF and 0,5μg ascorbic acid (E), demonstrating preservation of cell integrity and large aggregates (E-two panels on left) and extensive cell death (F-far right). Cells grown in presence of 1.5 μg of ascorbic acid (G) displayed numerous aggregates (black arrowheads) and cell little cell death. Cells that remained untreated (H) succumbed to contamination (H-left, red arrowhead) and displayed extensive cell death (H-right, yellow arrowhead)


A
B
C
D
20 µm
E
F
G
H

and cell division. Whether *in vitro* or *in vivo* several factors regulate the proliferation of cells. Growth factors that bind to receptors on the cell surface that connect to signaling molecules that convey message from receptor to the nucleus where transcription factors bind to DNA, turning on or off the production of proteins that cause cells to undergo mitosis. A variety of growth factors and molecules that interfere with the endogenous downstream signaling cascades have been characterized in *Platynereis* dumerilii. The presence of serum is routinely used in cell culture of other organisms, as it contains the molecules necessary for cell survival and growth factors necessary for cell maintenance and proliferation. Numerous studies have tried to characterize the factors present in Foetal Calf Serum or the hemolymph in the case of invertebrate models. Which of the factors are present in *Platynereis dumerilii* hemolymph is not known. A number of growth factors are commercially available and can be used as additives in cell culture. These constitute substances derived primarily from vertebrates and ecdysozoans. Search through the preliminary transcriptome database available to the lab revealed presence of transcripts encoding homololgs of vertebrate growth factors and their receptors. (Fig. 4.2.5)The presence of BMP and dpp has been documented in *Platynereis* [29]. The EGFR found to be present in *Platynereis* (Jekely *Platynereis* 454 Assembly Contigs contig17348). The contig obtained was confirmed as a *bone fide* EGFR transcript, as a BLAST of obtained sequence using the NCBI nucleotide database yielded transcripts encoding EGFR (top hits included: *Lymnaea stagnalis* Epidermal growth factor receptor (XM_002432299.1) with a sequence similarity of 99% E-value: 2e-52; *Pediculus humanus corporis* Epidermal growth factor receptor precursor, putative (XM_002411848.1) with a sequence similarity of 100%, E-value: 6e-52 and *Ixodes scapularis* Epidermal growth factor receptor, putative (XM_001602780.1) with a sequence similarity of 99%, E-value: 2e-51).

Therefore, these two growth factors were selected as possible candidates for cell culture testing.

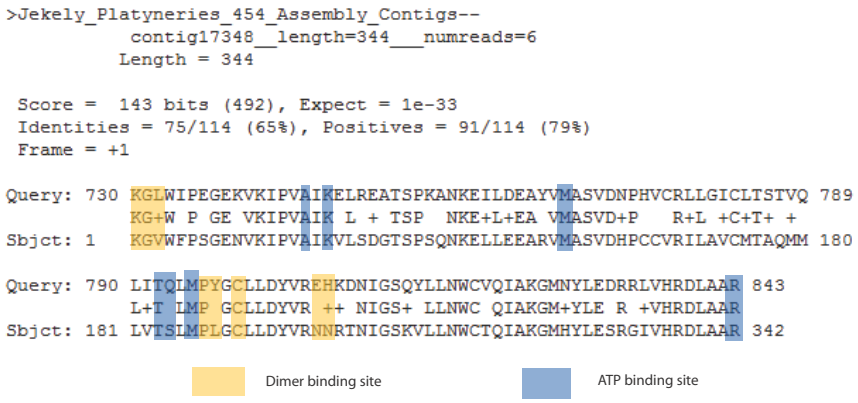


Figure 4.7: EGFR transcript in *Platynereis*

In order to determine the effect of supplements to this minimal medium, supple-

ments were added to individual 24-well plate wells and the effect observed over time. BMP4 was added on the 3rd day in culture. After 5 days in culture, wells containing BMP4 (Fig. 4.6A) contained larger aggregates as compared to control(Fig. 4.6B).After 23 days in culture, aggregates appeared to die. Aggregates appeared granular and individual cells were no longer observed. Extensive cell debris was observed. (Fig. 4.6C) However, dying aggregates possessed darker, (denser) cell aggregates on their surface that had not been observed earlier. Similar effects were observed in presence of EGF (incubated from day 5 in culture onwards, observed on day 23). Aggregates observed in presence of 5% EGF possessed similar denser cells(Fig. 4.6D) Individual cells had a granular appearance and cell membranes were irregular, indicating cell death. Cells grown in the presence of EGF and low concentrations of ascorbic acid did not display the dense cell clusters. Aggregates appeared similarly larger as after 2 days incubation with BMP4 (Fig. 4.6E). This effect could not be explained by the presence of ascorbic acid alone as incubation with medium concentrations of ascorbic acid had deleterious effects on cell survival (Fig. 4.6 F). Incubation at high concentrations of ascorbic acid resulted in the preservation of cell aggregates. Numerous individual cells were observed to adhere to plate surface and mildly adhering aggregates originally observed remained. (Fig. 4.6G). Cells grown in MM1 alone, were not as well preserved. Extensive cell death was observed. (Fig. 4.6H) Experiment was discontinued after 25 days due to conspicuous-looking growth in control wells (Fig. H Left). Growth was assumed to be of fungal origin although attempts to identify failed. Aggregates systematically remained in suspension and did not adhere to the bottom of the well plate. Individual smaller cells however, were observed to adhere to the bottom of the plate. Adhesion of smaller cells was observed independently of additives and only in 24 well plates. At no point was this observation made using 96 well plates or 6 well plates produced by the same manufacturer.

Due to limitation in the amount of starting material, quantitative assays could never be performed. In order to perform quantitative assays for periods spanning several months several dozen batches (simultaneously fertilized) would be necessary. As culture conditions did not allow this, all observations pertaining to Minimal Media were performed qualitatively only. Further inquiry into the requirements of *Platynereis* cells were rendered challenging due to the above described limitations. However, considerable research has been performed on the closely related *Nereis virens* nereid worm at Seabait Ltd, a company involved in breading *Nereis virens* for fishing bait. Seabait designed an method of improving the nutritional requirements of marine worms by feeding them a diet of concentrated pigments, polyunsaturated fatty acids, lipids, vitamins and/or minerals. Personal communication with Dr. Steven Craig (Seabait Ltd) stressed the importance of astaxanthin in the the diet of *Nereis virens*. Furthermore, Seabait Ltd used lyophilized or refractance window dried worms supplemented to aquaculture of adult and maturing worms. In developing the specialized feed, Seabait Ltd analyzed the nutritional content of the lyophilized feed, demonstrating a high variety of fatty acid methyl esters, a high composition of copper, phosphorus and vitamins C, A E and D3 [360].

4.2.6 Evaluation of the viability of *Platynereis* cells in commercially available media

A number of media elaborated for vertebrates and *Drosophila* are commercially available. They vary in their composition which was historically adjusted to that of vertebrate or insect serum or hemolymph composition.

Clad	Organisms	Cell type	Medium used	Supplemented with	Reference
Bryozoa	*Bugula nemerita*	ciliated cells	L15	D-glucose	Akano et al., 1999
Porifera	*Dysidea avara*	all cells	DMEM	-	Sipkema et al., 2003
	Negombata magnifica	all cells	L15	FCS	Rinkevich 1998
	Teichaxinella morchella	all cells	M199		Willoughby & Pomponi, 2000
Cnidarians	*Pocillopora damicornis*	all cells	DMEM	FCS	Domart-Coulon et al., 2001
	10 colonial cnidarians		L15, DMEM, M199		Frank et al,. 1994
	Sinularia flexibilis	all cells	DMEM, M199, L15, Grace's insect medium	FCS	Khalesi et all., 2008
	Acropora microphtalma	all cells	DMEM	FCS	Kopecky et al., 1999
Molluscs	*Ampularia*	dermatoid	M199	-	Chen, 2000
	Crassostrea gigas		L15	FCS	Domart-Coulon et al., 1994
	Calyptogena soyae		DMEM	FCS/ blood-like fluid	Koyama & Aizawa, 2000
	Pecten maximus	heart cells	L15	FCS	Le Marrec-Croq 1999
	Ostrea edulis	heart cells	L15, M199, BHK21	hemolymph Crassostrea and FCS	Renault 1995
	Crassostrea virginica	whole heart	JL-ODRP-4	-	Buchanan et al., 1999
	Pecten	digestive gland	L15	-	Le Pennec & Le Pennec 2001
	Ostrea eduls	heart & mantle	Seawater	-	Vago & Chastang, 1960
	Crassostrea virginica	mantle	-	Blue crab plasma	Perkins & Menzel, 1964
	Crassostrea virginica	Heart	M199 & seawater	-	Li et al., 1966
	Crassostrea virginica	Heart	Scherer's medium	-	Trip et al., 1966
	Spisula solidissima	Heart	MEM	-	Cecil, 1969
	Crassostrea virginica	Heart	2xM199	-	Brewster & Nicholson, 1979
	Crassostrea virginica	Hemocyte	MEM	-	Stephens & Hetrick, 1979
	Crassostrea virginica & Mya arenaria	Heart, gonad, mantle, palp, larvae & neoplastic hemocyte	NCTC-135, RPMI, L15		Hetrick et al., 1981
	Crassostrea virginica	Mantle & heart	MEM	-	Ellis et al., 1985
	Crassostrea virginica	Mantle & embryo	Ham's F-12	-	Ellis 1987
	Pinctada fucata & Pinctada magaritafera	Mantle & larvae	Medium Pf35	-	Machii & Wada, 1989
	Crassostrea & Mercenaria	Embryo	Ham's F-12	-	Ellis & Bishop, 1989
	Crassostrea virginica	Visceral ganglion	L15x2	-	Kleineschuster & Swink, 1992
	Crassostrea gigas	Heart	L15x2	-	Wen et al., 1993
	Meretix lusoria	Heart	L15x2	-	Wen et al., 1993
	Ostrea edulis	Heart	L-15 & seawater	-	Renault et al., 1995
	Crassostrea gigas		L15x2	FCS	Chen & Wang, 1999
	Haliotis tuberculata	hemocytes	M199	hemolymph	Lebel et al., 1996
	Mytilus edulis	digestive gland	L15	FCS	Birmelin et al., 1999
	Patinopecten yessoensis	mantle epithelium	L15x2	FCS	Endoh & Hayawa 2006
	Pecten maximus	digestive gland	L15x2	FCS	Le Pennec & Le Pennec, 2001
	Limulus polyphemus	amoebocytes	Grace's insect medium	amoebocyte lysate	Hurton et al., 2005
	Penaeus japonicus		M199	FCS	Lang et al., 2002
	Pinctata fucata martensii	mantle	BSS w/ glucose	-	Awaji & Suzuki 1998
Crustaceans	*Penaeus monodon*	lymphoid organs,	L15	cholesterol	Kasornchandra et al., 1999
	Penaeus chinensis	embryonic tissue	MPS	FCS, IGF-II, bFGF	Fan & Wang 2002
	Penaeus styliros		2xL15	FCS, EGF, interleukin-2	Tapay et al., 1997
	Penaeus styliros		2xL15	FCS	Shike et al., 2000
	Penaeus monodon	heart tissue, lymphoid organ, nerve, hepatopancreas	L15	FCS, prawn extract	Owens & Smith, 1999
	Penaeus monodon	ovarian tissue	Grace's medium, 2xL15	TPB, EGF, bFGF	Fraser & Hall 1999
	Nephrops norvegicus	hemocytes	2xL15	FCS, EGF, bFGF, IGF-I	Mulford et al., 2001
	Macrobrachium nipponense	muscle	M199	FCS	Wang et al., 2001
Ascidians	*Didemnun ternatum*		L15	FCS, insulin, VitE	Odintsova 1999
Echinoderms	*Asterias rubens, Ophiura ophiura*	neurons	L15, RPMI	coelomic fluid, sea squirt blood, NGF, FCS, radial nerve & stomach homogenate	Moss et al., 1998
	Asterias amurensis	embryonic archaenteron	Jamarin	glycin	Kaneko et al., 1995
	Strongylocentrotus purpuratus	mesenchyme	DM		Harkey et al., 1980

Table 4.3: Cell culture media used with marine invertebrate cells

Media component	DMEM	M199	RPMI	NEUROBASAL	L15	SCHNEIDER	F12
Amino acids							
Glycine	0.4	0.667	0.133	0.4	2.67	3.33	0.1
L-Arginine hydrochloride		0.332		0.398			
L-Arginine					2.87		1
L-Asparagine			0.379				0.1
L-Aspartic acid		0.226	0.15			3.01	0.1
L-Cystine 2HCl	0.201	0.108	0.208				0.2
L-Cystine						0.417	
L-Cysteine hydrochloride-H2O		0.000568					
L-Cysteine				0.26	0.992	0.496	
L-Asparagine-H2O				0.00553	1.89		
L-Glutamine	4	0.685	2.05		2.05	12.33	1
L-Histidine hydrochloride	0.2	0.104		0.2			0.1
L-Histidine			0.0968		1.61	2.58	
L-Glutamic Acid		0.455	0.136			5.44	0.1
L-Isoleucine	0.802	0.305	0.382	0.802	1.91	1.15	0.0305
L-Leucine	0.802	0.458	0.382	0.802	0.954	1.15	0.1
L-Lysine hydrochloride	0.798	0.383	0.274	0.798		9.02	0.199
L-Lysine					0.514		
L-Methionine	0.201	0.101	0.101	0.201	0.503	5.37	0.0302
L-Hydroxyproline		0.0763	0.153				
L-Phenylalanine	0.4	0.152	0.0909	0.4	0.758	0.909	0.0303
Serine	0.4	0.238	0.286	0.4	1.9	2.38	0.1
L-Proline				0.0675		14.78	0.3
L-Threonine	0.798	0.252	0.168	0.798	2.52	2.94	0.1
L-Tryptophan	0.0784	0.049	0.0245	0.0784	0.098	0.49	0.01
L-Tyrosine disodium salt dihydrate	0.398	0.221	0.111				0.0347
L-Tyrosine				0.398	1.66	2.76	
L-Valine	0.803	0.214	0.171	0.803	0.855	2.56	0.1
L-Proline		0.348					
β -Alanine						5.62	
L-Alanine							
Vitamins							
Choline chloride	0.0286	0.00357	0.0214	0.0286	0.00714		0.1
α-tocopherol Phosphate		0.0000142					
Ascorbic Acid		0.000284					
Biotin		0.000041	0.00082				0.0000299
D-Calcium pantothenate	0.00839	0.000021	0.000524	0.00839	0.0021		0.00105
Folic Acid	0.00907	0.0000227	0.00227	0.00907	0.00227		0.00295
Niacinamide	0.0328	0.000205	0.0082	0.0328	0.0082		0.000295
Pyridoxine hydrochloride	0.0194	0.000121	0.00485	0.0196	0.00485		0.000291
Pyridoxal hydrochloride		0.000123					
Para-Aminobenzoic Acid		0.000365	0.0073				
Riboflavin	0.00106	0.0000266	0.000532	0.00106			0.0000984
Riboflavin 5'-phosphate Na					0.000209		
Thiamine hydrochloride	0.0119	0.0000297	0.00297	0.0119			0.00089
Thiamine monophosphate					0.00226		
Menadione (Vitamin K3)							
Nicotinic acid (Niacin)		0.000203					
i-Inositol	0.04	0.000278	0.194	0.04	0.0111		0.1
Vitamin B12			0.0000037	0.000005			0.00103
Vitamin A (acetate)		0.000305					

Vitamin D2 (Calciferol)		0.000252					
Inorganic Salts							
Calcium Chloride ($CaCl_2$) (anhyd.)	1.8	1.8		1.8	1.26	5.4	0.299
Calcium nitrate (Ca(NO3)2 4H2O)			0.424				
Ferric Nitrate (Fe(NO3)3"9H2O)	0.000248	0.00173		0.000248			
Cupric sulfate (CuSO4-5H2O)							0.00001
Magnesium Sulfate (MgSO4) (anhyd.)			0.407				
Magnesium Chloride (anhydrous)				0.814	0.986		0.602
Magnesium Sulfate (MgSO4) (anhyd.)	0.814	0.814			0.814	15.04	
Potassium Chloride (KCl)	5.33	5.33	5.33	5.33	5.33	21.33	2.98
Ferric sulfate (FeSO4-7H2O)							0.003
Potassium Phosphate monobasic (KH2PO4)					0.441	3.31	
Sodium Bicarbonate (NaHCO3)	44.05	26.19	23.81	26.19		4.76	
Sodium Chloride (NaCl)	81.9	117.24	103.45	68.97	137.93	36.21	131.02
Sodium Phosphate dibasic (Na2HPO4) anhydrous			5.63				
Sodium Phosphate monobasic (NaH2PO4-H2O)	0.906	1.01		0.906		8.47	
Sodium Phosphate dibasic (Na2HPO4) anhydrous					1.34		1
Zinc sulfate (ZnSO4-7H2O)				0.000674			0.003
Other Components							
D-Glucose (Dextrose)	25	5.56	11.11	25		11.11	10.01
2-deoxy-D-ribose		0.00373					
D+ Galactose					5		
Ribose		0.00333					
α-Ketoglutaric acid						1.37	
HEPES	25.03			10.92			
Phenol Red				0.0736	0.0251		0.00319
Phenol Red					0.0266		
Sodium Pyruvate				0.227	5		
Sodium Acetate		0.61					
Glutathione (reduced)		0.000163	0.00326				
Hypoxanthine Na		0.00294					0.03
Thymidine							0.00289
Adenosine 5'-triphosphate		0.00165					
Adenosine 5'-phosphate		0.000576					
Adenine sulfate		0.0248					
Guanine hydrochloride		0.0016					
Thymine		0.00238					
Xanthine-Na		0.00224					
Uracil		0.00268					
Cholesterol		0.000517					
Linoleic Acid							0.0003
Lipoic Acid							0.00102
Putrescine 2HCl						0.001	
Tween 80							
Fumaric acid						0.862	
Malic acid						0.746	
Succinic acid						0.847	
Trehalose						5.85	
Yeastolate							

Table 4.4: Composition of commercially available cell culture media (based on the composition indicated on the Invitrogen website (concentrations expressed in μM)

A survey of literature revealed that the commercial media most frequently used for marine invertebrate cell culture attempts is Leibovitz L15 Medium single or double concentrated (Table 4.3). Double concentrated media are correspondingly not adjusted to higher osmolarity. Various media supplements have been used: purified compounds, mainly vitamins and non-specific nitrogens homologous hemolymph ; fetal calf serum, horse serum, yeast or bacterial extracts, extracts prepared from whole animals, or from specific tissues.

Based on this information, commercially available media (DMEM, L-15, L-15x2, F12, Neurobasal, M199, RPMI, Schneider, Optimem) were selected and the viability of cells incubated in each medium in presence and in absence of the additives investigated above was determined using the MTT-reduction assay. Leibovitz L15 medium has often been used in the double concentrated form as its osmolarity is then comparable to that tolerated by most marine organisms and therefore, addition of salt is not required. Therefore, additionally, cells were cultured in L15x2 solution. Frequent contamination by fungus and bacteria rendered a systematic comparison of cells cultured in each medium over time extremely challenging. It was, however, observed that cells grown in media lacking serum supplementation systematically displayed lower viability than those grown in identical conditions but in presence of FCS, independently of the stage of the larvae from which cells were derived. Several of the media tested are designed as synthetic media (M199, Schneider, Optimem) which means that they do not require serum supplementation for the culture of cells and tissues derived from their designated organism. However, the composition of each medium without serum was not sufficient for *Platynereis* cells. This indicates that additional factors, that are not present in each medium are required. Based on this observation and previous indications that FCS had a stronger effect on viability than hemolymph, all tests were therefore performed in presence of 10% FCS, unless otherwise stipulated. In this pilot experiment all cells reared in L15x2 solution showed signs of contamination after 1 day in culture. Cells derived from 12hpf, 24hpf, 48 hpf and 72 hpf larvae were obtained and resuspended in 100 μl of each media in 96 well plates. Due to systematic contamination, 12hpf and 48 hpf larvae-derived cells could not be analyzed quantitatively in all conditions tested. Cells obtained from 24hpf and 72 hpf old larvae also suffered from contamination and could not be analyzed reproducibly at a large scale after 3 days in culture. Using the MTT-reduction assay viability could only be evaluated quantitatively with all media tested in absence of visible contaminants at 48 hours in culture in this set up (n=3 wells). Incubation in MM1was used as a control (referred to as NSW in figure 4.2.6). Qualitatively, cells obtained from 12hpf larvae when not contaminated appeared round and did not show any visible signs of cell death for the first 3-5 days. Ciliated cells continued to "swim" for up to two days in culture. Rosette formation was observed during the first three to four days. Density and frequency of rosettes appeared diminished on days 4 and 5. Similar observations were established for 24hpf larvae-derived cells. Cell yields were higher in the sample of 24 hpf larva derived cells. Cells that did not suffer from bacterial and fungal contamination grew showed multiple morphologies. Most cells demonstrated a circular morphology. Cells with an elongated horn-shaped morphology were more frequent than in 12hpf larva-derived cells and maintained this morphology until day 4 or 5 in culture. Extensive cell death was observed after the fourth and fifth day independently of the medium

utilized. Cells in DMEM, RPMI, M199 and L15 systematically faired better than cells in MM1, F12, NB, Optimem and Schneider after day 4 or 5 in culture. Less cell death was observed in the first group of media than in the second. Cells derived from 48hpf larvae faired similarly with a stark decrease between day 3 and 4.5 in culture, in those wells in which contamination was not observed. 72hpf larvae however suffered from a decrease in cell viability after 2 days independently of the medium tested. Curiously, cells derived from 24, 48 and 72hpf old larvae incubated in Schneider medium were most severely affected cell death when it occurred whether in presence of in absence of contamination. At day 7 cells appeared practically all dead, with only a few (under 50 cells per well) left. Surprisingly, a plate forgotten in the incubator with these cells in Schneider medium (which was not supplemented with fresh medium for the duration of the incubation) was recovered after 2 months, and contained a small number (under 100) rosettes in the center of each well. The formation of these rosettes was not observed in wells containing other media.

Unlike the in the Minimal Medium experiment described above, media were not re-supplemented every three days due to fear of spreading contamination from one well to another. Thus it is possible that after three to four days in culture all nutrients necessary to cell survival were depleted and in absence of nutrients cells began to die. Thus second duplicate experiment with the exact same setup using cell derived from 24hpf old larvae was performed. The only difference was that every three days 50 μl of media were removed and an additional 50μl of freshly thawed medium were added for a period of one week. After this one week, all cells succumbed to contamination. However, in the interval before their visible irretrievable contamination the same pattern of cell death after 3 to 4 days was observed independently of the medium used. Due to limiting amounts of starting material quantitative evaluation of metabolism was not possible.

Based on these observations, it is tempting to hypothesize that an intrinsic program is followed by these cells. Early whole *Platynereis* larvae divide during the first couple of days and begin extensive differentiation starting at day 3. After 12hpf, few neurons are present. Thus cells placed in culture at an early stage of their development continue dividing in absence of differentiation cues and succumb to cell death after the intrinsic period 3 day cell division period. Cells obtained from larvae that have already undergone this 3 day division period *in toto* have a shorter portion of the programmed cell division period left and continue to divide in culture for a shorter period of time. At the end of this period during which, cells divide and suffer relatively low loss, cells begin to die. The media in which the cells were incubated affect this period most. Depending on the respective composition of the different media, cell metabolism is either accelerated or decelerated. Media such as RPMI, M199, DMEM and L15 are rich in sugars and vitamins and stimulate cell metabolism. Whereas, Schneider medium, which lacks vitamins all together does not, thus slowing cell metabolism after the initial period. Thus when MTT-reduction assay measurements are carried out on cells derived from older (72hpf larvae-derived) after two days in culture these cells have finished their extensive cell division program and their death is decelerated by less rich media such as MM1 and Schneider. Whereas, cells derived from younger larvae (12 or 24hpf old larvae) after two days in culture are still in their programmed proliferation phase. Thus the most rich media, such as M199, RPMI and L15x2 accelerate cell metabolism in

this phase whereas less rich media such as Schneider and F12 slow down metabolism. However, none of these media alone were capable of driving cell proliferation on the long term efficiently. For this reason, it was decided that additional supplementation was necessary. As remarkably often, successful primary culture of cells from marine invertebrates has been reported using the Leibovitz L15 medium, efforts were concentrated on supplementing this medium.

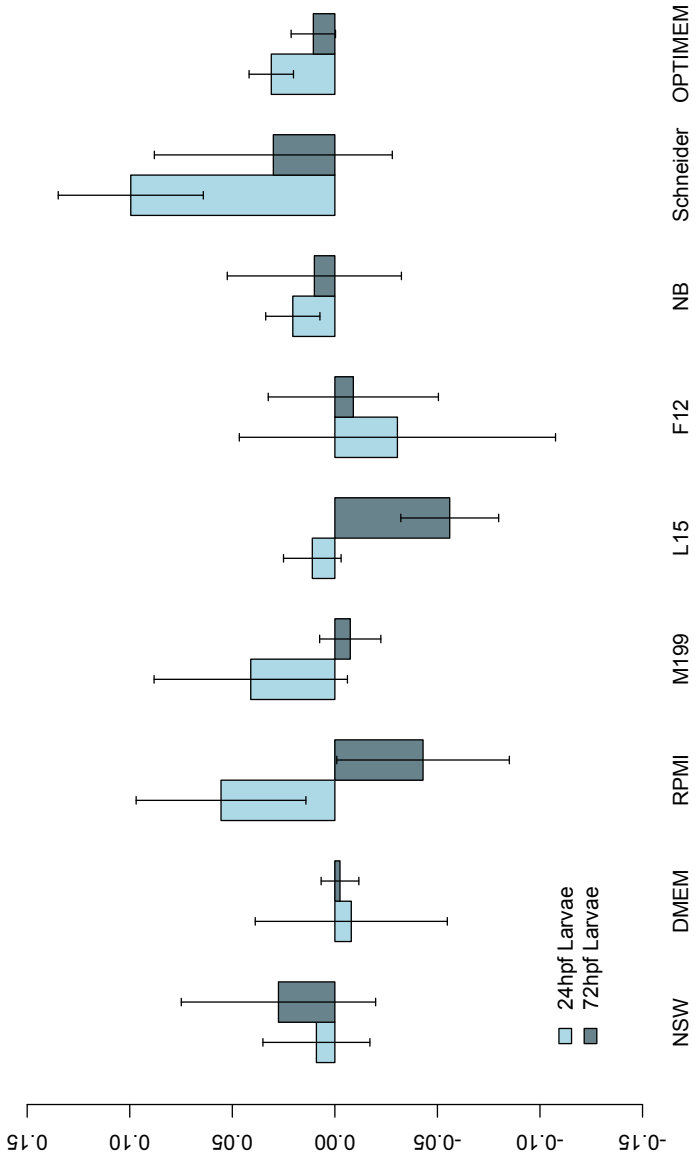


Figure 4.8: Viability of *Platynereis* cells in different media assessed using the MTT- reduction assay over a period of two days

4.2.7 Evaluation of the viability of *Platynereis* cells in supplemented commercially available media

Evaluation of the effect of α-tocopherol acetate, taurine and insulin on the viability of *Platynereis* cells

Considerable success has been achieved in the culture of mussel cells in a modified L15 medium by [361, 165]. This medium developed by Dr. Odintsova and Dr. Khomenko also contained α-tocopherol acetate (Vitamin E), taurine and insulin. Authors observed the differentiation of larva-derived cells into muscles and neurons only in this medium. Furthermore, muscle cells grown in this medium formed a network of contracting fibers. In an attempt to induce neurogenic differentiation, cells derived from young *Platynereis* larvae were cultured in different media containing the concentrations used by Khomenko and Odintsova of α-tocopherol acetate, taurine and insulin. Insulin is an important hormone involved in regulating glucose metabolism, whereas supplementation with taurine is based on the importance of taurine for mussel survival. Taurine (2-aminoethanesulfonic acid)is an organic acid derived from amino acid cysteine. It has been shown to be incorporated into low-molecular weight polypeptides found in the brain [362]. Marine polychaete *Glycera dibranchiata* was found to utilize taurine to reduce intracellular solute concentrations during hypoosmotic stress [363]. Hypotaurine accounted for 91% of free amino acid pool. Similar observations were made for the polychaetes *Hydroides elegans* and *Audounia Spirabranchus* in which taurine was demonstrated to be present extensively in muscle tissue [364]. Taurine and amino acids have been shown to accumulate in numerous marine invertebrates from seawater against gradients of 10^6:1 to 10^7:1 and have been proposed to be transported via specific sodium dependent transport systems. This may indicate that taurine may be important for the biochemistry of marine polychaetes, similarly to the situation encountered in mussel. α-tocopherol acetate is an important additive to cell culture media due to its capacity to prevent lipid peroxidation from oxygen radicals, a common problem to all aerobic cells. It acts as an antioxidant by donating a hydrogen atom to a lipid-peroxyl radical, making it a lipid-hydroperoxide whilst α-tocopherol is oxidized to a free radical. The tocopherol free radical, tocopherol can either (1) react with another lipid-peroxyl radical and become a stable product,(2) react with another tocopherol free radical to form a dimer, or (3) it can be regenerated by a reducing agent such as ascorbate. In the latter situation, the presence of α-tocopherol is maintained, allowing it to continue its antioxidant activity [365]. *In vivo*, α-tocopherol is found primarily in cell membranes. In serum, it is bound to and transported primarily by lipoproteins. Its physiological concentration in serum is typically in the range from 18 to 32 mM [365]. Furthermore, the taurine transporter was found to be expressed in *Platynereis* with maximum hits back to in the preliminary transcriptome data 4.2.7 : BAE80716.1 taurine transporter [Crassostrea gigas]5e-109 66% taurine transporter [Mytilus galloprovincialis] 7e-108 66% BAF95543.1 taurine transporter [Bathymodiolus septemdierum] 83% 6e-107 65% BAI66658.1 taurine transporter [Bathymodiolus platifrons] 83% 3e-104 63% .

Furthermore, α-tocopherol acetate is present in the basal composition of M199 (at a concentration of 6.7 nM) and F-12 (325 nM). L15 does not contain tocopherol. In-

```
>Sanger_Jekely_KN-1127_all_qual--KN-1127-B-93_J08_SP6.abi
          Length = 967

 Score =  277 bits (967), Expect = 8e-74
 Identities = 139/268 (51%), Positives = 183/268 (68%)
 Frame = +3

Query: 40  QREKWSSKIDFVLSVAGGFVGLGNVWRFPYLCYKNGGGAFLIPYFIFLFGSGLPVFFLEI 99
           +RE W++KIDF+L+  G  VGLGNVWRFPYLCYKNGGGAFL+PYF+ ++ +G+P FF+E+
Sbjct: 165 KRETWTNKIDFLLACIGFSVGLGNVWRFPYLCYKNGGGAFLVPYFLCVLVAGIPAFFIEV 344

Query: 100 IIGQYTSEGGITCWEKICPLFSGIGYASVVIVSLLNVYYIVILAWATYYLFQSFQKELPW 159
            +GQ+ S+GGI+ W K+CPLF GIG+A+ VI+ LLN+YY +IL WA YYLF SF   LPW
Sbjct: 345 ALGQFMSQGGISAW-KVCPLFQGIGFATTVIIFLLNIYYNIILTWAFYYLFSSFSAVLPW 521

Query: 160 AHCNHSWNTPHCMEDTMRKNKSVWITISSTNFTSPVIEFWERNVLSLSPGIDHPGSXXXX 219
           ++CN+ WNT +C   ++R +    +  +      PV E+WER VL +S GID  G+
Sbjct: 522 SNCNNEWNTENC-SRSVRDDNLTDLAFNRNTSVDPVTEYWERKVLQISTGIDDVGTIKWD 698

Query: 220 XXXXXXXXXXXXFFCIWKGVRSTGKVVYFTATFPFAMXXXXXXXXXXXXPGAGAGIKFYLY 279
                      +FCIWKG++++GKV+YFTAT P+ +           PGA  GIKFYL
Sbjct: 699 LALCLLLAWMVVYFCIWKGIKTSGKVMYFTATSPYILMCVLLIRGVTLPGAVEGIKFYLI 878

Query: 280 PDITRLEDPQVWIDAGTQIFFSYAICLG 307
           PD  RL D QVWIDAG QIFF Y+I LG
Sbjct: 879 PDWDRLRDTQVWIDAGNQIFFLYSISLG 962
```

Figure 4.9: Sequence of *Platynereis dumerilii* taurine transporter

sulin is commercially available as a solution of insulin only or as a supplement known as ITS. ITS is composed of 1 mg/ml insulin from bovine pancreas, 0.55 mg/ml human transferrin, and 0.5 μg/ml sodium selenite. ITS s a general cell supplement designed for use in non-complex media (MEM, RPMI-1640) and complex media (F-12, DME/F-12, MEM) with sodium pyruvate. In parallel to insulin supplementation, ITS supplementation was also tested.

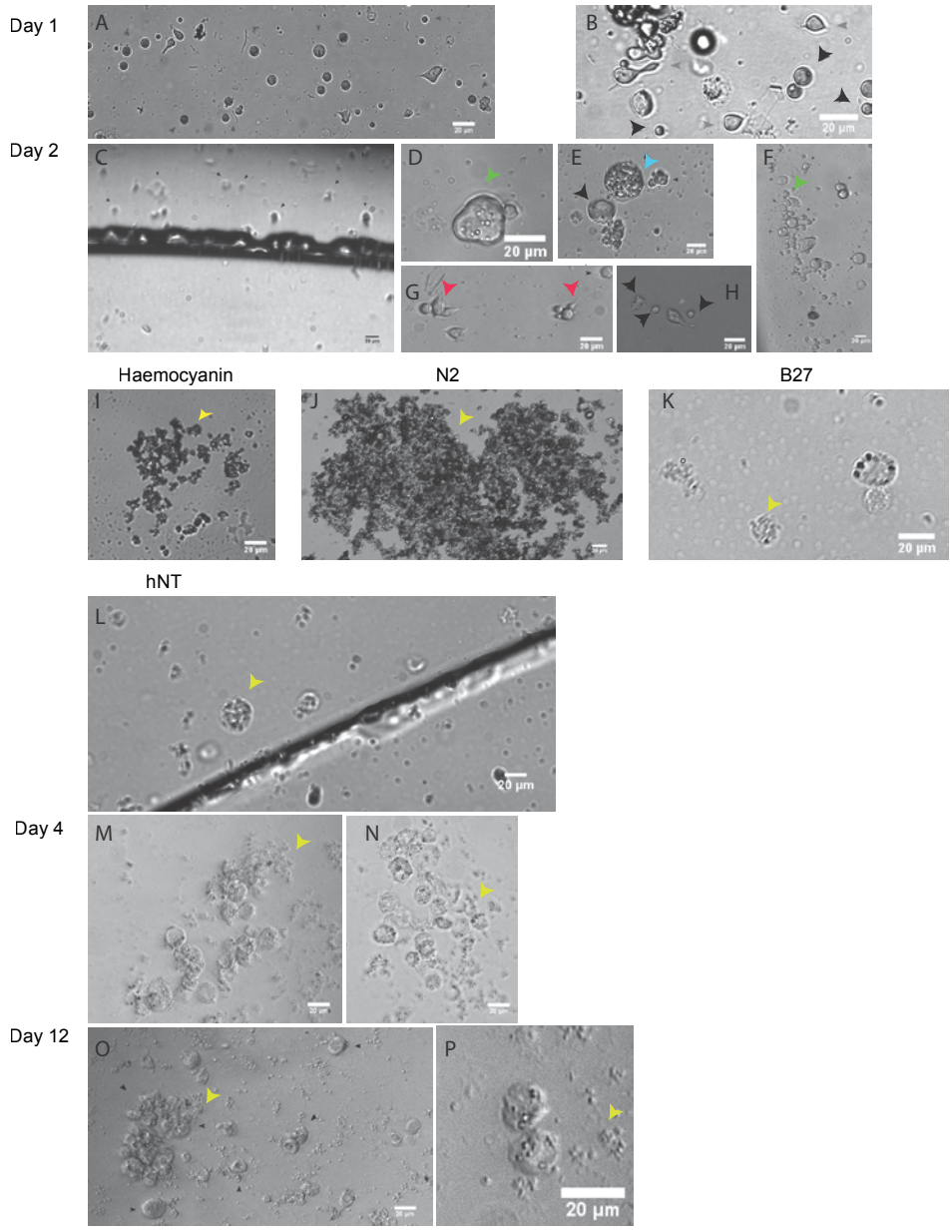
Evaluation of the effect of α-tocopherol acetate, taurine and insulin on the viability of *Platynereis* cells in L15 medium In order to determine whether each of these components exerted an effect on cells collectively and individually, media supplemented with only taurine and only insulin were used. However, α-tocopherol acetate is difficult to manipulate at high concentrations, because it is lipid soluble and must be resuspended in ethanol, which can be damaging to cells. To test whether ascorbic acid could help regenerate tocopherol, helping protect cells from oxidative stress, ascorbic acid was also tested (Fig. 4.10). Cells plated on in L15 medium in the presence of all three components (α-Tocopherol Acetate, Insulin and Taurine) were observed for a period of two weeks. After 13 days cells succumbed to fungal contamination. Presence of these three additives considerably increased cell adhesion selectively to glass surfaces (coverslips) as compared to 24 well plate surfaces (Black arrows in Fig. 4.10). Cell viability as in the experiments in the different media described above decreased after three to four days. Cell shapes were irregular and extensive cell debris was observed. Cells adhering to glass surface were less affected by this cell death event. These additives in the hands of Odintsova and Khomenko resulted in neuronal differentiation.

B27, N2 and hNT (Invitrogen) are standard supplements to vertebrate neuronal primary cultures. In order to determine whether these molecules can drive neuronal differentiation in *Platynereis* primary culture, cultures of cells in presence and in absence of α-tocopherol acetate, insulin and taurine. No visible difference was observed in absence and in presence of α-tocopherol acetate, insulin and taurine for treatment with B27 and hNT. In absence of α-tocopherol acetate, insulin and taurine no effect was observed in presence of N2. In presence of α-tocopherol acetate, insulin and taurine N2 appeared to exert a toxic effect on *Platynereis* cells. Cells died rapidly upon addition of the substance, and retained the aggregate formation. N2 medium supplement is composed of 1mM Human Transferrin, 0.08mM Insulin, 0.002mM Progesterone, 10mM Putresceine and 0.003mM Selenite [366]. It is supplied as a 100x stock solution. In all cases tested, this combination of substances was deleterious to *Platynereis* cells. Serial dilutions of the stock to 0.01x showed no toxicity, however, no effect was observed in regard to neuronal differentiation. Putative deleterious action is unlikely to be due to either of the substances tested as it is unlikely that any of these substances affect cells so rapidly. Instead, it is more likely that the cumulative pH upon addition of N2 was lowered to critical level. B27 and hNT (human neurotrophin) did not stimulate neuronal cell differentiation either. No toxicity was observed and addition at quantities of ranging from 50μl to 0.01μl did not rescue the cell death. No sign of neuronal differentiation was observed. The hypothetical cumulative acidity induced cell death was also observed upon treatment with other substances. Haemocyanins are respiratory metalloproteins that bind oxygen in molluscs and arthropods similarly to hemoglobin in vertebrates. Attempts to add Limulus derived hemocyanin (Sigma) to *Platynereis* cells at low concentrations had no effect. However at high concentrations resulted in a similar effect to that observed using N2, when the back bone of the cell aggregates remained but all the cells died within several dozen minutes.

In order to determine whether any of the components of the α-tocopherol acetate, insulin and taurine troika had an effect individually, each component was added alone at

---

Figure 4.10 *(following page)*: Effect of L15 Supplemented with α-Tocopherol Acetate, Insulin and Taurine. (A-B) Immediately after dissociation a number of individual cell types displaying different morphologies were observed. Some cells adhered to plastic (A) and glass(B)(Black arrowheads). 24 hours after plating, more cells were observed to adhere to plastic than to glass (C). Cells displaying non-circular morphologies were still observed (D-F, green arrowheads), including large cells containing numerous granular structures (blue arrowheads), unipolar cells (pink arrowheads) (G-H). Large non adhering aggregates containing numerous small cells (E) were observed. Adhering cells formed clusters. No cells were observed in wells incubated in presence of Shrimp Hemocyanin(I) after 48 hours in culture. Cell debris (yellow arrowheads) that retained the shape of the aggregate cells putatively derived from. Similar observations were made for cells incubated in N2 (J), B27(K) and hNT(L). Some cells survived in hNT. After 4 days in culture (M-N), cells displayed irregular cell membranes, presence of granular structures located unilaterally at one pole of each cell. After 12 days in culture(O-P), cell debris resulting from extensive cell death was observed. Adhering cell-like structures were abundant.(Black arrows) Adhering aggregates were prominent. Closer examination of cells (P) revealed the presence of dents in the cells and an increase in the size of intracellular granules as compared to those observed on day 4.


Day 1
A
B
Day 2
C
D
E
F
G
H
Haemocyanin
N2
B27
I
J
K
hNT
L
Day 4
M
N
Day 12
O
P
20 µm

varying concentrations and together over a period of two weeks. Attempts were made to evaluate cell viability both morphologically and using the MTT-reduction assay. Cells grown in L15 medium in presence of taurine alone (1μl per 100 μl culture in a 96 well plate), formed aggregates after two days in culture. These aggregates adhered to the 96well plate surface. (Fig. 4.2.7 Day2 +Taurine) On day three of culture aggregates were larger, composed of larger numbers of cells and containing larger cells (Fig. 4.2.7 Day3 +Taurine). On the fourth day of culture long chaetae-like structures were observed emanating from the cell aggregates(Fig. 4.2.7 Day4 +Taurine). Treatment with chitinase did not affect the presence of these structures indicating that they may not be chatae after periods of over 4 hours. This situation is remarkably different from that observed in the absence of α-tocopherol acetate, insulin and taurine described above, in which most cells began to die after 3-4 days in culture. By the end of the first week of culture large cells resembling muscles that did not contract were observed. All aggregates sunk to the bottom of the well and some cell debris become visible(Fig. 4.2.7 Day7 +Taurine). By day 9 extensive cell death was observed. Few cells remained transparent and displayed a healthy appearance, within aggregates. These cells were mainly located on the inside of the aggregates rather than on the outside. Addition of insulin to the same concentration of taurin (ITS supplement, Sigma) appeared to deleteriously affect cell health on the second day, with large amounts of cell debris. This effect was attributed to high low pH. However, on the next day extensive aggregates were visible. (Fig. 4.2.7 Day3 +Taurine+Insulin). By the fourth day of culture cells reared in presence of taurine and insulin displayed a marked increase in the amount of cells, similarly to the situation observed with taurine alone, and differently from the situation described previously in which both these substances were absent and in which α-tocopherol acetate, insulin and taurine were present together (see different media comparisons above). At the end of the first week of culture, cells became contaminated with fungi and the observation was discontinued. Cells incubated in taurine, insulin and 0.5 μg ascorbic acid (vitamin C) displayed structures resembling chatae that were previously observed in treatment with taurine alone on the fourth day (Fig. 4.2.7). Cell aggregates were much larger on the third day of culture in these conditions, resembling the situation observed in presence of taurine alone (larger cells within non-adhering aggregates) and larger sized aggregates containing more cells. These aggregates split into smaller aggregates by the fourth day of culture and larger cells were visible by the end and middle of the first week of culture. On day 10 of culture cells were observed to be contaminated by fungi. SImilarly cells cultivated in presence of taurine, insulin and 1 μg ascorbic acid formed large aggregates that grew to large sizes (Fig. 4.2.7 right-most column).

Based on these qualitative observations, quantitative evaluations were attempted. For this purpose, cells were plated in 96-well plates and α-tocopherol acetate, insulin, taurine, ascorbic acid and EGF. The effect of EGF is known to be strongly dependent of the other components of media present in the culture. For this reason, the effect of EGF was evaluated alone, in presence of insulin and ascorbic acid and taurine. Cells plated in presence of EGF alone after one day in culture showed a lower cell viability than in its absence (Fig. 4.2.7A). Contamination was observed on the second day of culture and therefore this assay was discontinued. In presence of insulin the effect of EGF in L15 medium was the same as in its absence. This assay however did not

succumb to contamination and demonstrated a temporary increase viability on day two of culture followed by a decrease on the third day (Fig. 4.2.7E). This decrease was also observed presence of ascorbic acid and ITS (Fig. 4.2.7C and D). In presence of ascorbic acid and insulin however, this decrease in viability was recovered after five days in culture (Fig. 4.2.7H). This trend was not observed in presence of taurine and in absence of insulin(Fig. 4.2.7K). Despite frequent supplementation after 25 days in culture the viability readings observed at the beginning could not be recovered(Fig. 4.2.7L). Based on these observations, it was concluded that EGF was did not positively affect cell proliferation and viability, whether in the presence or in the absence of other additives including ascorbic acid, insulin, ITS and α-tocopherol acetate.

Attempts to compare the effect of insulin and ITS systematically over time failed due to recurrent contaminations. However, from the experiments that were not contaminated it can be concluded that overall in all the combinations tested the effect of ITS was negative. Cell viability as determined by the MTT-reduction assay in presence of the ITS reagent was judged to be diminished as compared to any of the other conditions tested in the same assay (Fig. 4.2.7B, C, D, F and L). Insulin in absence of transferrin and selenite in all conditions tested did not appear to decrease viability. A trend similar to that in absence of any additives was always observed, cells in the presence of insulin independently of other additives added, increased viability during the first two to three days and then sharply decreased in viability(Fig. 4.2.7A, D, E, F, G, H, I and M). This trend is altered in the presence of EGF (Fig. 4.2.7H and L) where viability increases after five days, and in the presence of very high concentrations of ascorbic acid (over one μg)(Fig. 4.2.7I, J and K). Thus, none of the results observed by Odintsova and colleagues on mussel cells could be reproduced using *Platynereis* cells. Neither muscle formation, nor neurogenesis was stimulated by the addition of α-tocopherol acetate, insulin and taurine. Furthermore, neither alone nor in combination with other promising candidates, ascorbic acid and EGF, did these additives stimulate cell survival and proliferation as judged by a qualitative and a quantitative evaluation. Instead, ascorbic acid and EGF were observed to be more efficient at maintaining cells and stimulating cell larger aggregate formation.

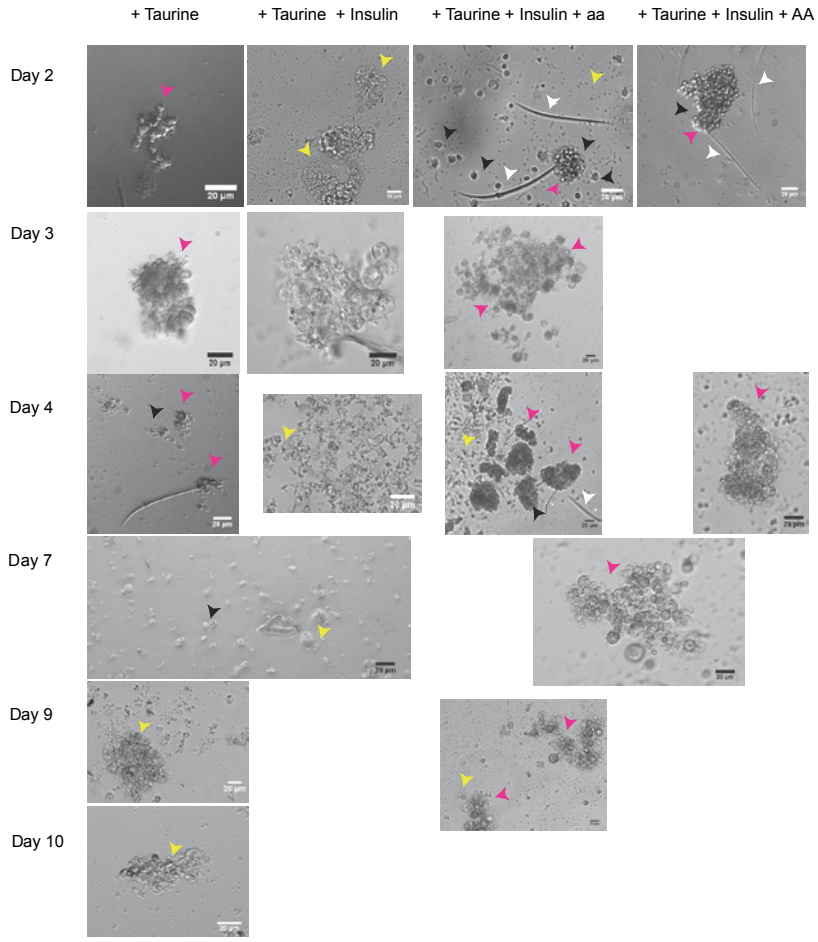


Figure 4.11: Effect of Insulin, Taurine and Ascorbic acid supplementation of L15 medium. Incubation of cells in L15 medium in presence of Taurine alone after 48 hours revealed the presence of small cellular aggregates (red arrowheads). These cells did not display any sign of cell death. Cell contours were even and little cell debris was observed. Cells incubated in presence of Taurine and Insulin exhibited extensive cell death (yellow arrowheads). Cell debris was prominent, some retained the shape of the putative aggregate from which they hypothetically derived. Cells incubated in presence of Insulin, Taurine and 0, 5μg ascorbic acid exhibited less cell than than in absence of ascorbic acid (B)but more than in absence of insulin Numerous adhering aggregates (black arrows) were observed surrounding chaetae (white arrows) (NB: cells were derived from 4dpf-old larvae). Cells incubated in presence of 1μg formed numerous large aggregates associated with chaeta (white arrows).

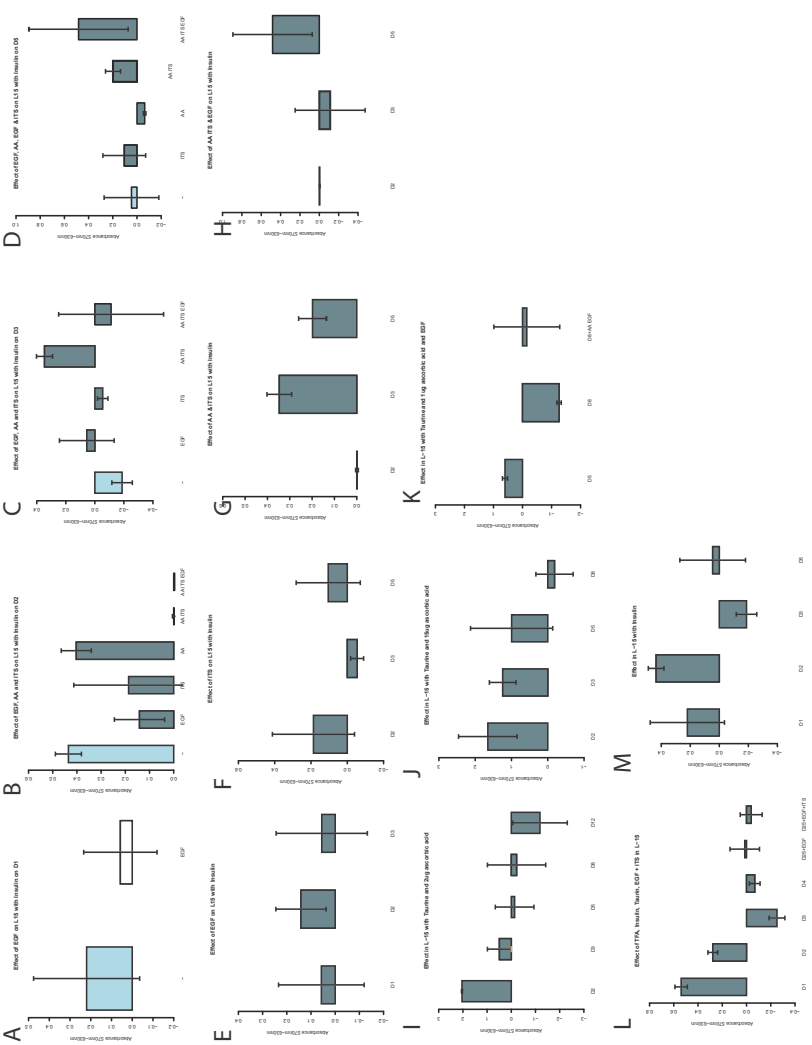


Figure 4.12: Quantitative Evaluation of the Effect of Ascorbic Acid, Taurine, EGF and ITS in L15 medium over three days in culture

Evaluation and comparison of the effect of α-tocopherol acetate, taurine and insulin on the viability of *Platynereis* cells in different media However, growth factors and hormones are known to act synergistically with other media and sera components. Therefore, the effect of ascorbic acid and ITS, and of EGF was tested in cells cultured in the different media. As observed previously, cells demonstrated the highest viability in rich media such as L15 (single and double strength)(Fig. 4.2.7B, C, G, I, J and M), M199 and DMEM, but low viability in Neurobasal, Optimem, MM1 and RPMI (Fig. 4.2.7B, C, G, I, J and M). When in presence of low amounts (0,1 μg) the effect of ascorbic acid in combination with the same amounts of ITS(x1) and EGF (1%), the cumulative effect was not clear (Fig. 4.2.7C), however when in presence of higher amounts of ascorbic acid (1μg) (Fig. 4.2.7D)the cumulative effect of the three substances added appeared to follow a similar trend to that observed in absence of EGF (Fig. 4.2.7B). Cells cultured in L15 (single and double strength), M199, F12 and DMEM displayed significantly higher viability that in Neurobasal and RPMI. Cells cultured in different media in presence of EGF (Fig. 4.2.7 E) displayed considerably lower viability, as compared to cells in presence of ascorbic acid and ITS (Fig. 4.2.7 B-C). However, a similar trend was observed where, cells cultured in Schneider, L15 and M199 displaying a higher viability that in MM1. The viability of cells grown in all the other media could not be measured as they succumbed to fungal contamination. An examination of the effect of ascorbic acid, EGF and ITS on cells cultured in each medium was then performed. Addition ascorbic acid, ITS and EGF had no significant effect upon viability of cells grown in 2xL15(Fig. 4.2.7A). Cells grown in presence of EGF had a considerably higher viability than those in grown in presence of ITS and ascorbic acid in Schneider medium. Ascorbic acid had a deleterious effect on cell viability. This could be explained by the fact that Schneider does not contain a good buffering capacity and that ascorbic acid is highly acidic. Cells may have died due to acidity of medium. Cells grown in presence of ascorbic acid, ITS and EGF exhibited a lower viability than in their absence in RPMI medium. Presence of EGF resulted in cell death. (Fig. 4.2.7D) Negative values indicate that absorbance read from control was indistinguishable or lower than that of sample containing cells, indicating extensive cell death. Cells grown in MM1 were not significantly affected by the presence of ascorbic acid, ITS or EGF. Cell viability was low in all conditions observed. Cells cultured in DMEM medium, were not significantly affected by the presence of ascorbic acid, ITS and EGF. In presence of EGF, cells displayed slightly lower viability. Cells cultured in L15x1 medium responded significantly negatively to the presence of EGF alone and no significant effect to EGF was observed between cells cultured in presence of ascorbic acid and ITS. Cells cultured in Neurobasal medium responded significantly negatively to the presence of ITS and EGF. Cells cultured in F12 displayed dosage dependent response to ascorbic acid in the presence of ITS. Presence of EGF in media containing ITS and 0.5μg ascorbic acid did not have a significant effect (Fig. 4.2.7).

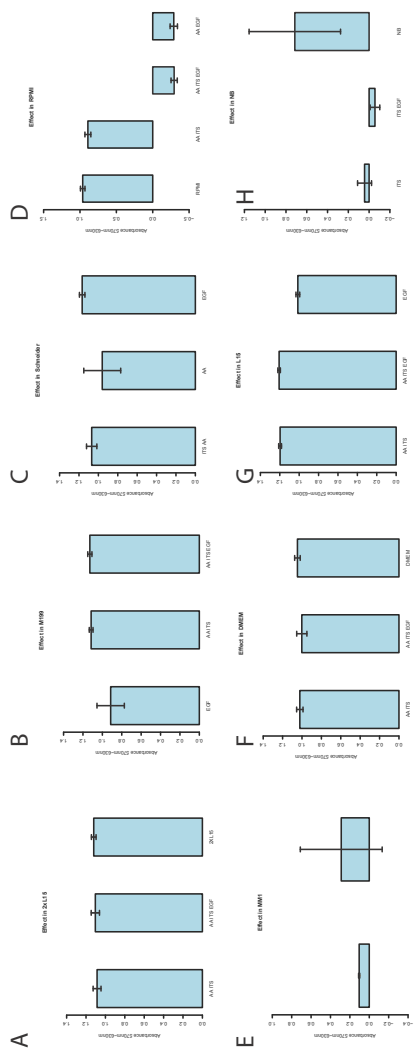


Figure 4.13: Quantitative Evaluation of the Effect of Ascorbic Acid, Taurine, EGF and ITS different media In (A) 2x L15 medium (B)M199 medium (C) Schneider medium (D) RPMI (E) Minimal medium (F) DMEM (G) L15x1 (H) Neurobasal medium (I) Ham's F12 medium

Evaluation of the effect of α-tocopherol acetate, taurine and insulin on the viability of *Platynereis* cells in Schneider medium Supplementation cells grown in Schneider medium with α-Tocopherol Acetate, Insulin and Taurine demonstrated higher viability as evaluated by the MTT-reduction assay as compared to cells obtained from a similar stage reared in the absence of these supplements (Fig. 4.2.7 top panel). Schneider medium does not contain any vitamins and low amounts of energy sources. Addition of insulin may have affected metabolism and addition of α-tocopherol acetate may have aided membrane integrity.

Evaluation of the effect of α-tocopherol acetate, taurine and insulin on the viability of *Platynereis* cells in M199 medium Supplementation cells grown in Schneider medium with α-Tocopherol Acetate, Insulin and Taurine resulted in the trend observed in non-supplemented media. After several days a stark decrease in viability as determined by the MTT-reduction assay was observed. This decrease could not be remedied by additional supplementation with 0.5μg ascorbic acid or 0.1% EGF. The difference between

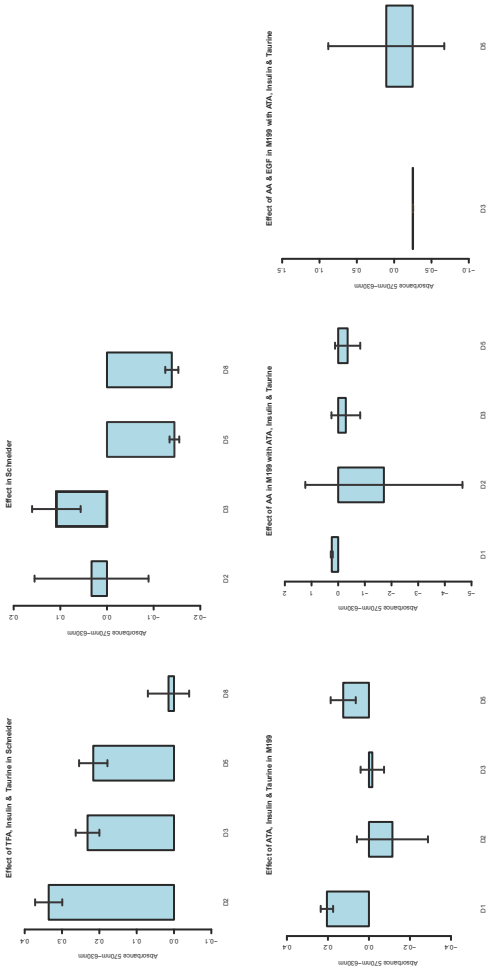


Figure 4.14: Quantitative Evaluation of the Effect of α-Tocopherol Acetate, Insulin and Taurine in Schneider and M199 media Top panel : Viability of *Platynereis dumerilii* cells as determined by the MTT-reduction assay cultured in the Schneider medium in the presence of α-Tocopherol Acetate, Insulin and Taurine (left panel) and in their absence (right panel) over a period of 8 days in culture Bottom panel: Viability of *Platynereis dumerilii* cells as determined by the MTT-reduction assay cultured in the M199 medium in the presence of α-Tocopherol Acetate, Insulin and Taurine (left panel), supplemented with 0.5 μg ascorbic acid (central panel) and supplemented with 0.5 μg ascorbic acid and 0.1% EGF

Evaluation of the effect of ascorbic acid, EGF and ITS on the viability of *Platynereis* cells in M199 medium Cells cultured in M199 in the presence of ITS (1μl per 96 well plate well in a total volume of 100μl) did not display a visible difference. Neither cell number nor cell morphology appeared to be affected. Addition of 1%EGF did appear to affect cells either. This was confirmed using the MTT-reduction assay after 4 days in culture. Addition of ascorbic acid (0.5μg) did not affect the MTT-reduction assay readout, however cell clusters were observed to display a more healthy morphology. Cell aggregates were larger and remained for the most part in suspension. Adhering cells grew larger and smaller cells were observed to shed off of the clusters after the third day in culture. After 7 days in culture an increase in the amount of cell debris was observed (top panels at 7 dpp in Fig. 4.2.7). The morphology of the remaining cells was also severely affected. Cells were no longer circular but slightly elongated or smaller. Increasingly, small black clusters became visible within the cells. However, other groups of cell aggregates within the same wells were observed to increase in size and smaller cells were observed to be shed off of these aggregates. An increasing proportion of cell aggregate were observed to grow in suspension or adhere to the well plate bottom. Cells sunk to the bottom but adherence was rarely observed. These cells survived for over a month and finally succumbed to fungal contamination (bottom panels at 7dpp in Fig. 4.2.7).

4.2.8 Investigation of the feasability of *Platynereis* explant culture

As described in the Introduction to this chapter, in the early XXth century cells utilized for culture were not obtained by dissociation in early cell culture experiments. Proteolytic treatment to obtain cell was not common. Instead, cells were obtained from explant cultures. Portions of organs and body parts were dissected from the organism investigated and placed in culture conditions. The culture of nereid explants has been previously attempted by Durchon and Schaller in 1964. The authors cultured *Nereis virens* parapodia in a solution containing agar, seawater, glucose, albumin and horse or chicken serum, obtaining tissue survival for up to one month. In order to determine whether the media conditions tested for cells could be amenable to *Platynereis* tissue and explant culture, adult organisms were dissected and pieces tissue were placed in culture in the different media described above in presence or in absence of supplements. Initial dissection was performed in collaboration with Antje Fischer. Explants of parapodia, head, stomodeum (Fig. 4.2.8) and caudal regions were isolated. To avoid contamination from marine protozoa, bacteria and fungi, worms were dissected in the presence of 3 drops Unimarine/L of SFNSW. Moreover, collected tissues were incubated in Penicillin and Steptomycin. Dissections were carried out on a box of ice under a binocular. Attempts were made to start explant cultures are listed in Table 4.2.8.

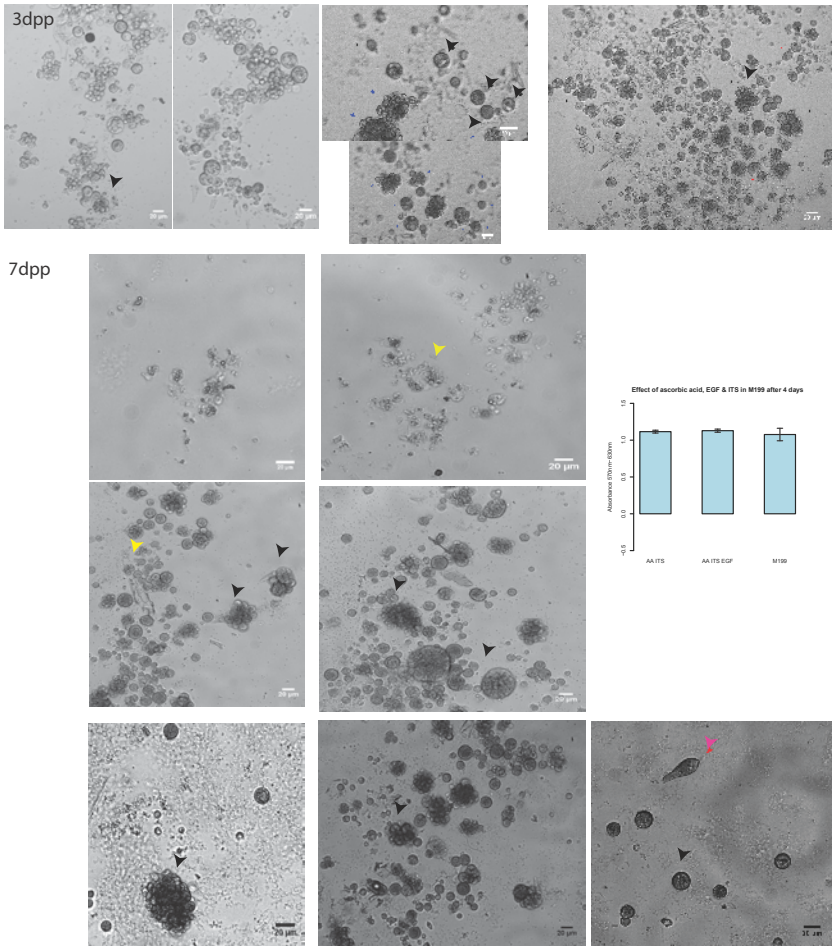


Figure 4.15: Qualitative and quantitative evaluation of the effect of ascorbic acid and ITS in M199 medium. Cell aggregates - black arrowheads, cell debris - yellow arrowheads, specialized morphology - red arrowhead

Tissue	Survival	Cells obtained
Parapodia	Survived for up to 3 months (n=37/52)	muscle-like cells, numerous elaeocytes, large quantities of large circular cells
Whole head	Survived for up to 3 week before succumbing to contamination (n=6/48)	generally small cells, no neuritic projections, muscle-like cells (no contraction)
Portions of head	Survival for 1 week	only small circular cells
Stomodeum	Survival for up to 3 months (n=43/53)	muscle like cell, numerous circular cells were shed, extensive amount of elaeocyte-like cells, extensive mucous-like substance
Ventral nerve cord	No sign of contamination for 3 weeks	No neuritic outgrowth
Gut	Frequent contaminated unless treated with ethanol	idem as for stomodeum

Table 4.5: List of attempts carried out to obtain explant cultures from adult *Platynereis dumerilii*

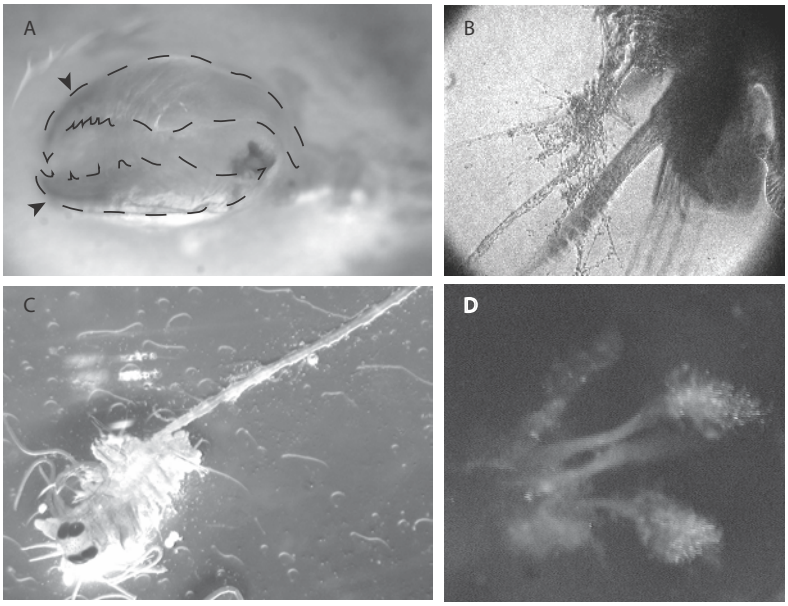


Figure 4.16: Starting material used from *Platynereis* explant culture: (A) Stomodeum (arrows indicate jaws) (B) Parapodia (C) Ventral nerve cord (D)Close up of cellular protrusion emanating from ventral nerve cord

Specificities of culture of adult tissue Curiously, *Platynereis* explants containing portions of the trunk were practically never contaminated with bacteria. These could be attributed to the presence of large amounts of coelomocytes and elaeocytes in the hemolymph and interstitial fluids. These macrophage-like cells can phagocyte foreign bodies. Moreover, media changes were not required frequently. As described above, dissociated cells derived from larval tissue required fresh medium addition every 3 days to remain alive and healthy. Adult tissue could be left untouched for several weeks without resulting in extensive cell death. It could be perhaps postulated that the remaining tissue integrity of the organs cultured was sufficient to provide the necessary cell-to-cell signals to maintain cells alive. Moreover, paracrine and endocrine growth factor secretion cannot be excluded. The best results were obtained using parapodia. If decontaminated first, by spraying with ethanol, as well as treatment with Unimarine and Penicillin/Streptomycin, non-contaminated cells could be obtained. As time passed, less cells would be visible within the parapodial explant, and more cells would be visible migrating out of the explants in waves. Contaminations were frequently observed to migrate out of the explants. If cultures were performed in 24 well plates, cells morphologically resembling protozoa were frequently observed. However, if the decontamination treatments and subsequently the culture were carried out in tis-

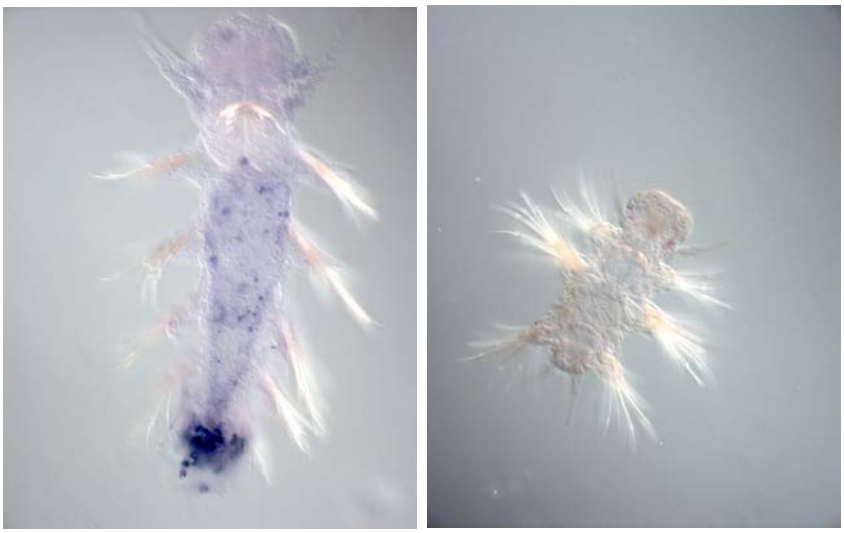


Figure 4.17: Phosphohistone H3 labelling on two week old *Platynereis* larvae (left) staining the posterior growth zone and no primary antibody control (right).

sue culture flasks, which can be sealed shut, contamination was rarely observed. Early XXth century cell culture enthousiasts, used spinning cultures. In order to increase the detachment of cells from the explants and to migrate away into ”cell culture”, flasks containing the cells were place on a rotating device. I attempted a similar setup using a spinning wheel (curtesy the Spitz lab). However, no increase in cell viability as judged by morphology nor increase in the number of cells escaping explants was observed. A larger proportion of cells remained in suspension as compared to cells cultured in a stationary fashion. As many cells grew specifically only in suspension, an attempt to maintain all cells in suspension was tested, by placing a magnetic stirrer in the tissue culture flask. This culture did not prove to be more successful than cultures lacking magnetic stirrers.

A further interesting particularity of *Platynereis* adults and older larvae is the presence of stem cells in the caudal-most region of the their body, dubbed the posterior growth zone. Cells located in the region are capable of regenerating a damaged or missing caudal feature. Due to the pluripotent nature of these cells, I attempted to enrich such cells in culture. In order to determine their localization within several week old larvae, a phospho-histone H3 staining was performed to label dividing cells (cells undergoing S phase to be more precise)(Fig. 4.17), inline with previously published observations [367, 35, 368].

Larvae aged 5 day to several weeks were embedded in low melting temperature agarose dissolved in SFCMFSW on ice to minimize muscle contraction and ciliary beating. Attempts to immobilize larvae with 3.5% magnesium solution were not effi-

cient in my hands. The caudal portions of larvae were cut off using scalpels and immediately placed in an ice-cooled solution of minimal medium. Approximately 320-330 pygidia were thus dissected and attempts to culture them were carried out over a period of two and a half weeks. Some cells were shed, however, extensive proliferation was not observed. On the contrary, extensive cell death was observed as with dissociated cell culture after 2-3 days in culture. Regular supplementation of medium to cells did not affect the outcome of the culture. On day 13 of culture, fungal contamination was observed and culture attempts were discontinued.

Co-culture of cells of different origin

The origin of cells used for culture can influence the success of culture. Larval and embryonic cells are totipotent and are easier to maintain without contamination than cells derived from feeding older larvae and adults. Culture of embryonic cells alone in DMEM invariably resulted in cell death or contamination within several weeks to a month. Younger larvae also express growth and differentiation factors that may be absent in older larvae. In order to determine whether a combined culture of cells derived from larvae different stages could affect the outcome of the culture several attempts were carried out co-culture cells of different stages. Co-culture of cells obtained from 4 dpf larvae with 40 hpf larvae resulted in rapid obtention of large dense tissue fragments which grew in suspension in DMEM and small-sized adherent cell aggregates which never reached the sizes of the dense tissue fragments in suspension. (see Fig. 4.18). Smaller aggregates adhered to non-coated glass coverslips as well as Nunc 24-well and 96 well plates. It was concluded that cells were grown in absence of serum demonstrating that dissociated larval cells from later stages maintain the capacity to secrete growth factors capable of either inducing aggregation or cell division in cells derived from younger larvae. The dense suspension aggregates were similar in morphology to adult tissue - see section - explant culture. Addition of zebrafish BMP4 (Fig. 4.19) accentuated this effect. However cells succumbed to contamination quite rapidly and effect could not be further investigated.

The effect described can be likened to that of conditioned media. In a parallel experiment, media from adult *Platynereis* (DMEM) head culture was supplemented in a culture of 24-hpf old larvae-derived cells in culture for one week. Cells were left alone with further manipulation for a supplementary week. On day 16, a surprising observation was made. Cells had formed a large mass of cells that was contracting. The contractions were not synchronized and with different portions of aggregate contracting at different times, pulling the entire structure in different directions. Addition of fresh media, in one of the wells, resulted in a cessation of all contractions. Of the 7 wells in which this observation was carried out, 3 succumbed to contamination. The remaining contracting cells were fixed and stained with DAPI and phalloidin, confirming that the aggregated structures indeed corresponded to muscle tissue (Fig.4.20).

4.2.9 Attempts at transfection of *Platynereis cells*

Functional interference is possible in *Platynereis* and has been successfully achieved in the lab. Microinjection of fertilized oocytes has rendered possible the transient

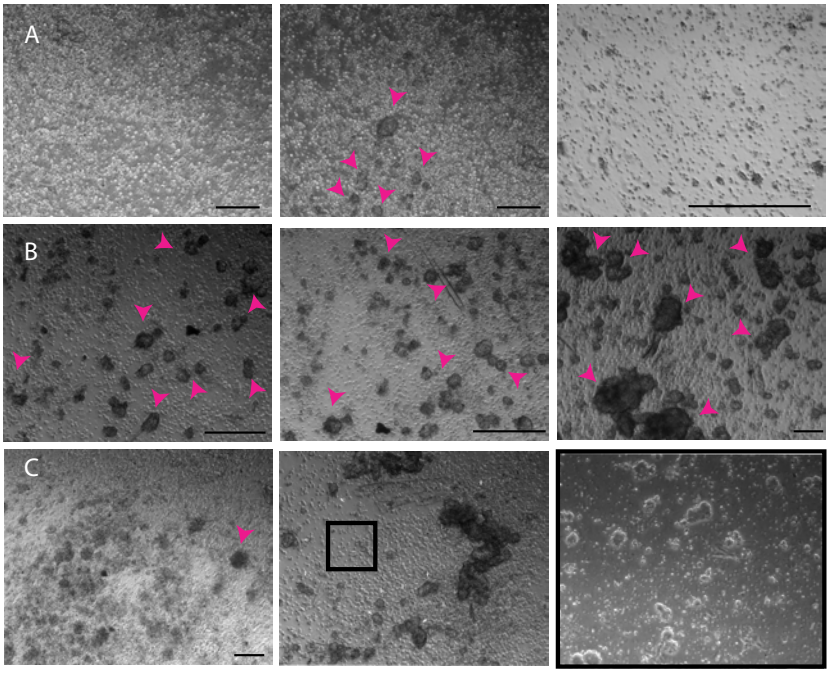


Figure 4.18: Results of co-culture of cells derived from 4-dpf old larvae with cells derived from 40-hpf old larvae. (A)Culture of 40hpf old larvae derived cells (B)Co-culture of cells derived from 4-dpf old larvae with cells derived from 40-hpf old larvae after 1-2 days in culture(C) Co-culture of cells derived from 4-dpf old larvae with cells derived from 40-hpf old larvae after 4 days in culture (red arrowheads indicate cell aggregates)

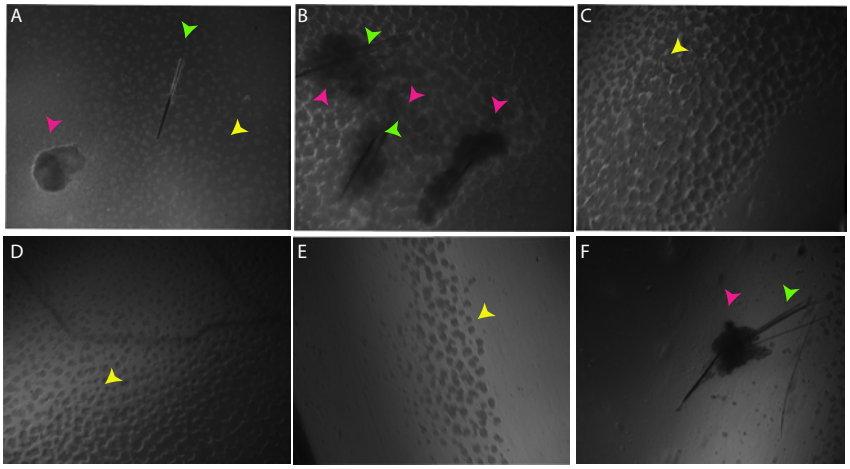


Figure 4.19: Results of co-culture of cells derived from adult *Platynereis* with cells derived from 40-hpf larvae in presence of BMP4.(A-C)Explant culture of adult cells in the presence of BMP4 (D-F)Co-culture of adult cells and 40hpf old larvae derived cells (Red arrowheads - explants, green arrowheads - chaetae, yellow arrowhead - adhering cell clusters)

and stable expression of reporter genes. Transient knock downs have been achieved using siRNA and morpholino injections. These tools can be powerful, however in order to obtain transfected cells for cell culture, several whole batches need to be injected, which is chronologically infeasible. Transfection of cells with expression vector pGRN145 (ATCC MBA-141) containing the human telomerase reverse transcriptase gene (hTERT) has ”worked to some extent in primary sponge cell cultures”. Sponge cell aggregates termed primmorphs can also produce a form of this protein resulting in difficulty distinguishing between the effects of the vector and the endogenous protein (Personal communication Jim Grasela, Harbor Branch Oceanographic Institute, Florida Atlantic University, Florida, USA). To make use of such a tool, it is first necessary to be able to either transfect large amounts of larvae or embryos or to transfect the dissociated cells themselves. With this goal in mind, several attempts were carried out to transfect *Platynereis* embryos, larvae and dissociated cells.

At first efforts were concentrated on electroporation of larvae with the PCS2+ CMV-GFP plasmid (obtained curtesy of the Wittbrodt lab, EMBL Heidelberg and Ruprechts-Karl University, Heidelberg). Electroporation were carried out as recommended by the siPORT siRNA Electroporation Protocol(Ambion). Electroporation involves applying an electric field pulse to induce transient cell membrane permeability and the formation of microscopic pores in the cell membrane allowing molecules, ions, water and nucleic acids to traverse the membrane. Experiments were performed on 24hpf larvae and 5 day old larvae. Electroporation was carried out at 150, 300, 450, 600, 750 and 900V with a pulse length of 0.1 ms. No fluorescence was observed over a

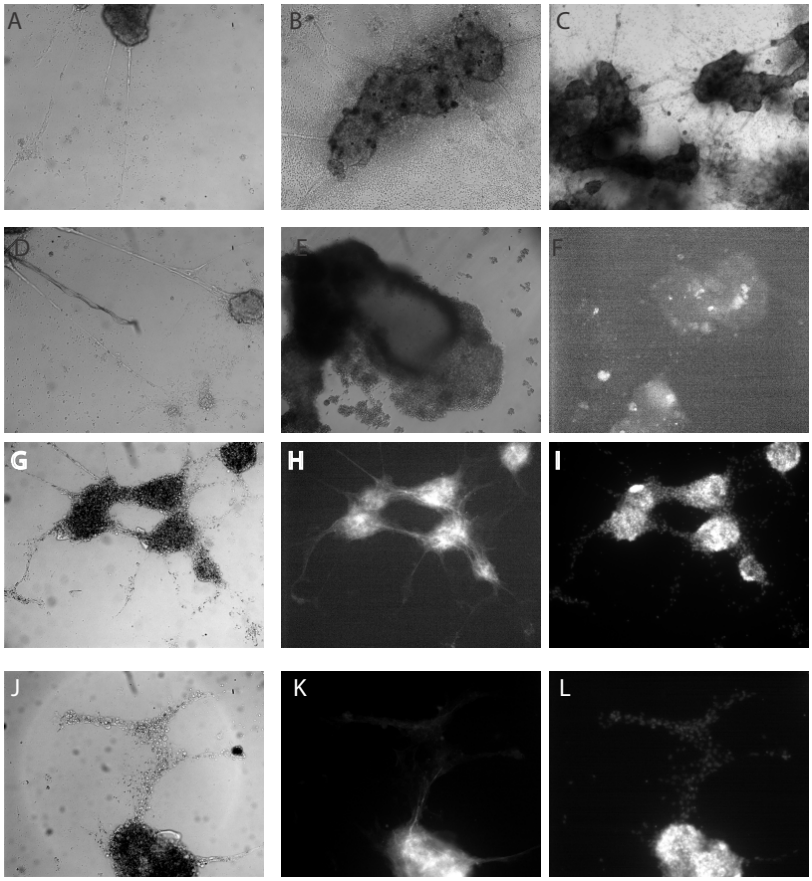


Figure 4.20: Evaluation of conditioned media from adult *Platynereis* head and stomodeum on 24-hpf trochophore larval cells in culture for two weeks, by DAPI and phalloidin staining(A-E) The different morphologies observed in the contracting large aggregates. (F) Autofluoresence in the GFP channel (G-L) Staining of fixed previously contracting aggregates with phalloidin (H and K)and DAPI (I and L) (G and J) display the transmission image without staining

period of 24 hours. For this reason larvae were treated with proteinase K at 100 mg/ml for 1 minute. The same experiment was repeated. No fluorescence was observed over a period of 24 hours. Addition of mannitol to compensate for the salt concentration was recommended in order to ameliorate electroporation efficiency in marine organisms (Personal communication H.Snyman)[369]. The same experiment was repeated, however no fluorescence was observed over a period of 24 hours. Attempts to electroporate dissociated cells, were also unsuccessful, cells adhered to the cuvette surfaces and considerable loss in cell quantity was observed. Cells in contact with mannitol solution underwent osmotic shock and died. As extensive cell death was observed it was impossible to distinguish between autofluorescence produced from dying cells and fluorescence due to GFP expression. Control electroporations lacking plasmid also displayed a signal in the GFP wavelength. After several attempts, this approach was abandoned.

In collaboration with Dr. Mette Handberg-Thorsager, Ms. Antonella Lauri (Arendt lab, EMBL Heidelberg) and Mr. Christian Hoerner (Hufnagel group, EMBL Heidelberg) attempts were made at transfection of cells using lentivirus. Lentiviral preparation and infection was carried out by Christian Hoerner in an appropriate S1 facility. Autofluorescence was observed several times, however within the scope of my collaboration no successful infection was observed. Infection attempts were carried out on dissociated cells, young embryos and larvae. My contribution to this collaboration consisted of providing dissociated cells in cell culture media.

Moreover, a third approach was tested, using lipofection. Lipofectamine 2000 was employed as described by the manufacturer (see Materials and Methods). PCS2+ CMV-GFP at various concentrations was used for transfection. Tests were carried out in freshly plated cells and cells in culture for 24 hours. As lipofection is not recommended in presence of serum, initial tests were carried out in the recommended Optimem medium. However, cells did not survive in this medium. Subsequent tests were carried out in DMEM, RPMI, L15 and 2xL15 media, in absence and presence of serum. No fluorescence was observed in any of the trials attempted.

Using a fourth approach, a coating strategy was attempted in collaboration with Dr. Beate Neumann (Advanced Light Microscopy Facility, EMBL Heidelberg). This approach has previously been used in high-content RNAi screening by time-lapse fluorescence microscopy of live HeLa cells expressing histone-GFP to report on chromosome segregation and structure [370]. 96 well plates were coated in presence and absence of fibronectin with 200, 400 and 800ng/μl of PCS2+ CMV-GFP or Ubiquitin-GFP in Lipofectamine 2000. DNA trapped in the cationic lipid droplets was then lyophilized using a speed vacuum. This method has the advantage of allowing adhering cells to absorb DNA directly, in situations where lipofection may not work due to high salt concentrations and allows the usage of serum in the culture medium. To verify whether this method works, fertilized oocytes and dissociated cells from young larvae were plated onto the coated plates. No fluorescence was observed in any of the wells tested and examined.

Substrate	Cells adhere	Cells remain in suspension
Gelatin	N	Y
Laminin	N	N
Poly-L-Lysine	(Y)	Y
Carbon coating Y	Y	
Arg-Gly-Asp-Ser (RGDS) - peptide	N	Y
Arg-Gly-Glu-Ser (RGES)-peptide	N	Y

Table 4.6: Adhesion assay on *Platyenereis cells*

4.2.10 Cell Adhesion assay

One reason for lack of success in obtaining differentiation and proliferation of cells is that cells were not plated in a confluent fashion and did not adhere to the surface of the dishes used. It is well established that differentiation occurs when cells adhere to a solid substrate [371]. Integrins are transmembrane receptors that can interact with extracellular matrix (ECM) proteins and can be largely responsible for cell adhesion and differentiation to specific cell types [372]. Odintsova demonstrated that the Arg-Gly-Asp-Ser (RGDS)-peptide, an inhibitor of integrin receptors and cell adhesion was could affect myogenic differentiation in mussel cells. RGDS-peptide blocked cell adhesion and inhibited myogenic differentiation, whereas incubation of the cells with control RGES-peptide did not affect myodifferentiation [165].

As the adhesion properties of different cells vary, the choice of substrate to grow cells on was important. Different marine invertebrate cells have been described to adhere to a variety of substrates. Therefore, cell adhesion was tested in the presence of different factors and the cell viability was catalogued. The RGDS and RGES peptides utilized by Odinstova were also tested [165] (Table 4.6). As mentioned previously, cells were observed to adhere to untreated coverslips more readily than to plastic. Particularly strong adhesion was observed to Nunc 24-well plate and 96 well plate surfaces rather than to Nunc 6-well plates. Gelatin coating prevented cells from adhering. All cells remained in suspension. Laminin treatment of glass coverslips did not substantially affect adhesion as compared to non-coated coverslips. Cells sunk to the bottom of the wells but no anchorage was created. Gently agitation could displace the cells easily. Coating of coverslips with poly-L-lysine resulted in some cells adhering more strongly to the coverslips than to control non-coated coverslips. This was rarely observed in large aggregates. Small cell rosettes were observed to adhere. However large circular aggregates rarely adhered to the surface of the coated coverslip (Table 4.6). Carbon coating showed the highest rate of cell adhesion. Coverslips treated with this method, however impeded cell staining as extensive washes in buffers containing detergents carried excess carbon particles and provoked unspecific cell antibody staining.

Odintsova's RGDS and RGES-peptide adhesion test was performed. Cells did not appear to adhere more readily to either substrates as compared to non-coated glass substrate. No difference in cell morphology was observed, indicating that cell rosette formation and cell adhesion in *Platynereis* early trochophore cells is not dependent on integrins (Table 4.6).

Increasingly, traditional cell culture methods are becoming replaced with microflu-

idic based setups in which small scale concentration fluctuations can be controlled. Frequently, these setups are made in a PDMS matrix. In order to test whether *Platynereis* cells could grow on such an environment, cells were grown on a petri dish coated with PDMS or a bubble of media containing an explant of interest was injected into the PDMS before it had finished polymerizing. Indeed, *Platynereis* cells grew on and into the PDMS substrate from the bubble explants. Cells grown on the suface of the PDMS did not appear to adhere to the matrix or enter it by any means. The presence of different media, did not affect the explant cells' capacity to invade the matrix. Cells grown in DMEM were equally capable of invading the PDMS as cells grown in Schneider.

4.2.11 Confirmation of the origin of the cultured cells method development

Due to very frequent contamination, a method needed to be devised in order to ascertain that the cells in culture were indeed derived from *Platynereis*. As cultures were carried out in volumes of 100 μl. RNA extraction from whole wells did not always make it possible to recover RNA or DNA from the contaminants. For this reason, effort was concentrated on growing the contaminants. Upon advise from Dr. Elia Benito-Gonzalez marine contaminants were grown on agar supplemented with Guillard's solution in petri dishes covered with a low amount of SFNSW(Fig. 4.21). In this way contaminants could be plated onto an agar substrate and fungi and bacteria could be grown in sufficient quantities for identification and different contaminants could be separated one from another. The next step to identification was to lyze the contaminating cells and determine their nature. Lysis using , heating to $60\,^{\circ}\mathrm{C}$, $70\,^{\circ}\mathrm{C}$ and $90\,^{\circ}\mathrm{C}$ was inefficient at extracting DNA for colony PCR. Instead proteolytic treatment was attempted. Treatment with Proteinase K, Pronase and Zymolyase were attempted. Zymolyase at 60U/ml was observed to successful lyze fungal contaminants.

4.2.12 Media determination for *Nereis virens* cells

In the past, several attempts have been made to design media *Nereis virens* cell and tissue culture. Durchon and Schaller cultured *Nereis* parapodia in a solution containing agar, seawater, glucose, albumin and horse or chicken serum, obtaining tissue survival for up to one month [373]. Heacox studied the protein and salt composition of *Nereis* hemolymph and elaborated the 'Nereis Balanced Salt Solution' (NBSS) for the culture of *Nereis* oocytes. NBSS is loosely based on commercially available vertebrate M199 medium, supplemented with commercially available amino acids and vitamin solutions defined for vertebrate needs (MEM Vitamins and Amino Acids). Heacox observed that supplementation of NBSS with vitellin had the most potent effect on oocyte growth and survival in vitro as compared to serum-based supplements.

In the present study, an approach analogous to Durchon and Schaller was adopted, by first testing cell growth in a minimal medium containing commercial amino acids and vitamins, supplemented with Foetal Calf Serum (FCS), *Nereis virens* hemolymph or *Nereis virens* coelomic fluid at concentrations varying from 2% to 10% [373].

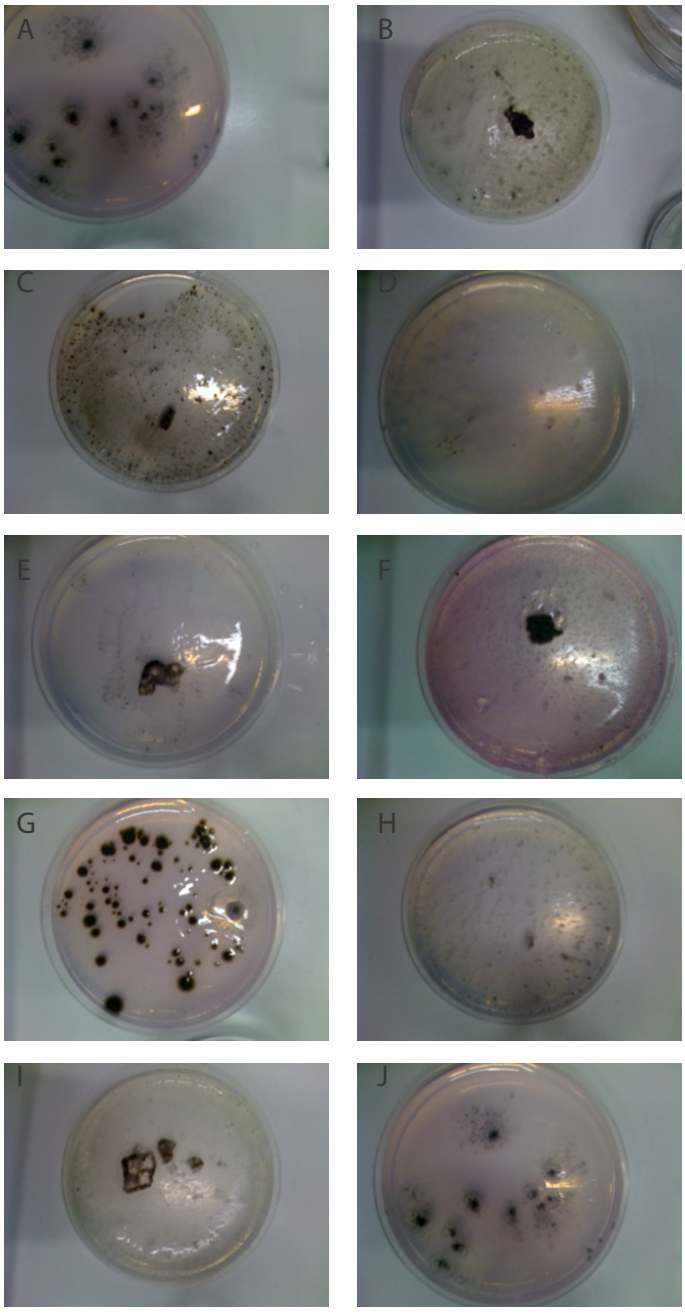


Figure 4.21: *Platynereis* and*Nereis virens* contamination grown on agar supplemented with Guillard's solution. (A-F) Fungal contaminants from culture of adult *Platynereis* (G-J) Fungal contaminants from culture of adult *Nereis virens*

Method used	Lysis	PCR
Heat lysis at 65 °C for 10 minutes in presence of Tween 20	-	-
Heat lysis at 65 °C for 20 minutes in presence of Tween 20	-	-
Heat lysis at 75 °C for 10 minutes in presence of Tween 20	-	-
Heat lysis at 75 °C for 20 minutes in presence of Tween 20	-	-
Heat lysis at 65 °C for 10 minutes in presence of 5% NP-40	-	-
Heat lysis at 65 °C for 20 minutes in presence of 5% NP-40	-	-
Heat lysis at 75 °C for 10 minutes in presence of 5% NP-40	-	-
Heat lysis at 75 °C for 20 minutes in presence of 5% NP-40	-	+/-
Freeze-thaw lysis at −20 °C for 10 minutes	-	+/-
Freeze-thaw lysis at −20 °C for 20 minutes	-	+/-
Freeze-thaw lysis at −80 °C for 10 minutes	-	+/-
Freeze-thaw lysis at −80 °C for 20 minutes	-	+/-
PK 100μg/ml for 20 minutes	-	-
Pronase 5% for 20 minutes	-	-
Zymolyase 10Ufor 10 minutes	+/-	+/-
Zymolyase 25U for 10 minutes	+/-	+/-
Zymolyase 60U for 10 minutes	+/-	+/-
Freeze-thaw lysis at −80 °C for 20 minutes followed by heating at 75 °C and Zymolyase 60U for 30 minutes	+++	+++

Table 4.7: Establishing lysis conditions for marine fungal contaminants

Serum requirements of *Nereis virens* cells At high concentrations of hemolymph (10%), extensive cell debris was observed. Large crystalline precipitates were observed after 1 day in culture, indicating an incompatibility between the hemolymph, amino acid solutions and vitamin solutions and concentrated cell and tissue suspension. At lower concentrations of hemolymph(5%), less cell death was observed as compared to 10% hemolymph medium. Large aggregates of lipid droplets and cells were observed floating on the surface of the medium. Cells and lipid droplets were also observed adhering to Nunc plastic 24 well plates and non-treated glass coverslips.

Cell death in 10% hemolymph medium can be explained in terms of osmolarity. The formation of crystals in 10% hemolymph should have affected the osmolarity of the medium. As *Nereis* cells are iso-osmotic, the osmotic shock may explain the extensive cell death observed. As coelomic fluid corresponds to dilute hemolymph in terms of solute content, the factor that precipitated in 10% hemolymph may have been present in 10% coelomic fluid but at lower concentrations, limiting the amount of precipitate formed. The 10% FCS medium did not form precipitates, indicating that the factor responsible for crystal formation is specific to *Nereis* fluids.The cell death in all conditions observed demonstrate that a minimal media containing MEM vitamins and amino acids, supplemented with growth factors derived from *Nereis* or calf are not sufficient for maintaining and inducing *Nereis* cell proliferation.

NB: all cells were obtained from a single maturing *Nereis virens*, hence samples (elaeocyte or dissociated cells and tissue) were equally exposed to contaminants. When

cells perished by succumbing to bacterial contamination in one medium but not in another, the medium in which cells died was judged inadequate for the maintenance of elaeocyte and coelomocyte antibacterial function.

***Nereis virens* cells in DMEM** DMEM is a rich medium. Dissociated densely plated *Nereis virens* tissue and cell aggregates adhered to coverslips and plastic in DMEM 5% /textitNereis virens haemolymph however they did not adhere to in DMEM in absence of serum additives. Elaeocyte cell suspension died after 3 days in DMEM in absence of serum due to bacterial proliferation. An unknown component of DMEM was judged to be non-permissive for antibacterial action of coelomocytes. Whether it is a component that is necessary and absent from media or a component exerting an inhibitory action on elaeocytes was not clear from this experiment. The densely plated cells grew as well in presence of hemolymph as in its absence, demonstrating as with *Platynereis* tissue culture that large tissues are capable of secreting a factor that maintains cells alive independently of medium additives.

***Nereis virens* cells in Schneider** Elaeocyte cell death was observed in Schneider in absence of serum. This is not surprising as Schneider is a very poor medium which does not contain any vitamins. Furthermore, Schneider contains yeastolate a protein component commonly used for bacterial culture, thus enhancing conditions for bacterial survival. Surviving *Nereis* cells adhered to plastic and glass. Dissociated tissue and cells adhered to plastic and glass in absence and in presence of 5% *Nereis virens* hemolymph. As mentioned for DMEM - plating density had a stronger effect than presence of serum.

***Nereis virens* cells in L15** L-15 medium is commonly used for the culture of marine invertebrates. Some success has been achieved with this medium for the culture of mussle cells in presence of insulin, α-tocopherol acetate and taurine. Taurine is important in mussle cells. Jorgensen and Kristensen have demonstrated that *Nereis* whole worms readily take up taurine from seawater [374].Elaeocytes survived in L-15 in absence of hemolymph and did not succumb to contamination. The density of elaeocytes was higher in medium containing insulin, indicating that insulin affects elaeocyte viability. Cells were observed in suspension and adherent to plastic. Dissociated cells however, died. Both aggregates in suspension and cells that adhered initially, by day 4 post-plating were absent. Survival was weak, however slightly higher in presence of hemolymph.

***Nereis virens* cells in RPMI** Similarly, cells cultured in RPMI in presence of Taurine, Insulin and α-tocopherol acetate did not exhibit cell death for several weeks when plated at high density Fig. 4.22B). Diluted cells, plated at a density ¡1000 000 cells/ml were supplemented with ascorbic acid. Using the MTT-reduction assay(Fig. 4.22A), it was observed that viability was increased in presence of low concentrations of ascorbic acid (100μg) but decreased at high concentrations (500μg)

Due to limitations in starting material and numerous contaminations, most culture tests were performed on *Nereis virens* elaeocytes or cultures enriched in elaeocytes and

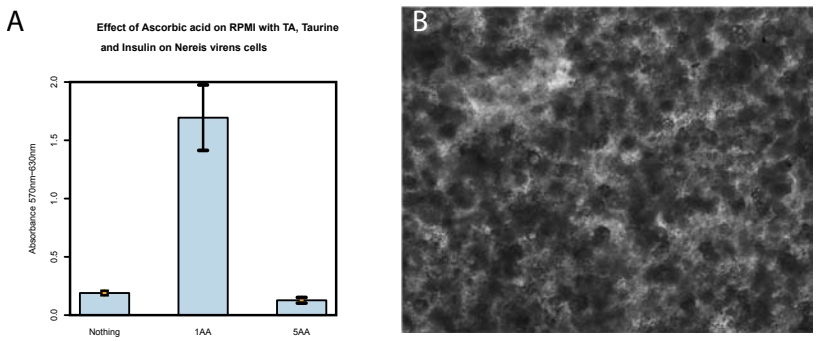


Figure 4.22: Effect of RPMI supplemented with Taurine, Insulin and α-tocopherol acetate in presence or in absence of ascorbic acid on the culture of *Nereis virens* cells.(A)Effect of supplementation with ascorbic acid determined using the MTT-reduction assay(B)Cells grown in presence of Effect of RPMI supplemented with Taurine, Insulin and α-tocopherol acetate

therefore, from here on referred to as elaeocyte culture, although non-elaeocyte cells may have been present in cultures.

Media determination for the culture of *Nereis virens* Elaeocytes

Elaeocytes cultured in 10% coelomic fluid also suffered from cell death, however precipitates were not observed (Fig 4.23). Minimum medium containing 10% FCS likewise exhibited extensive cell death and an increase in contaminant proliferation. No precipitates were observed. Aggregates of lipids and cells were observed adhering to 24well plate surfaces.

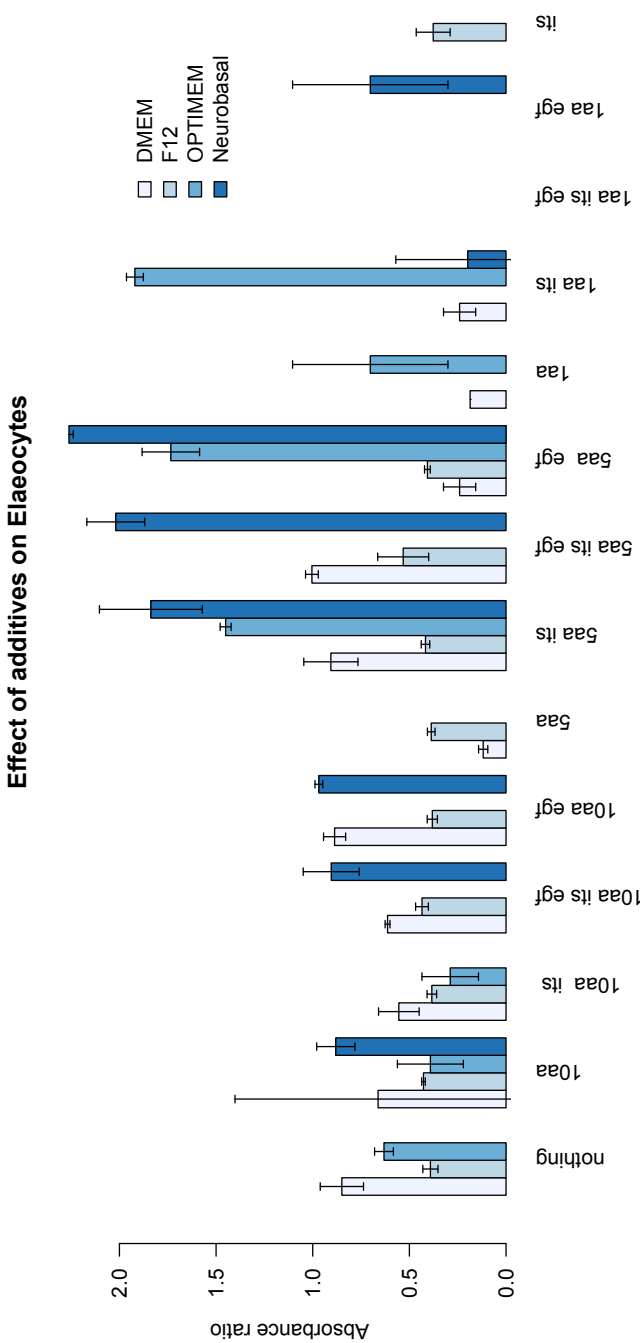


Figure 4.23: Effect of additives on elaeocytes grown in DMEM, F12, OPTIMEM and Neurobasal.

Elaeocyte culture in F12 medium Elaeocytes perished in F12 medium in absence of serum. Few elaeocytes remained, some in suspension. Empty clear spots were visible at bottom of dish. Hypothetically, this may be due to phagocytic activity of elaeocytes. This phenomenon was only observed in cultures in which massive cell death had occurred and no cells in suspension were present to obscure empty spots. As mentioned above, plating density influenced cell survival more than the media in dissociated cells with tissue clumps present. Similar results wer observed with Optimem and Neurobasal media in absence of serum.

Due to the success of mussel cell culture in presence of α-tocopherol acetate and taurine in L-15 and short-lived success for *Platynereis* cells in RPMI supplemented with the above mentioned components, *Nereis* cells were cultured in RPMI. Elaeocytes perished both in suspension and adherent, indicating that as with DMEM and F12 either a factor necessary for elaeocyte activity is absent or an inhibitory factor is present. Densely plated dissociated tissue and cells survived.

Elaeocyte culture in M199 medium The Heacox medium mentioned above is based on M199 medium. Surprisingly, cells did not survive well in M199 in my experience, indicating that the requirements of *Nereis* oocytes vary greatly from those of other *Nereis* cells. *Nereis virens* hemolymph was expected to be necessary and sufficient for cell growth and survival.

Very few cells survived in suspension. Adherent tissue and cells were dying.In presence of M199 and FCS cells also died. Few aggregates with lipidic structures remained in presence of FCS, insulin, taurine and tocopherol acetate.

Elaeocyte culture in Neurobasal medium In Neurobasal medium, elaeocyte-like cells rapidly died, leaving only cell debris and few lipid-droplet-like structures. Within 24 hours the characteristic vacuolar structures were absent and became replaced with cell debris(Fig. 4.25A. The effect of supplementation with ascorbic acid, EGF and ITS was examined using the MTT-reduction assay. Original attempts using the CellTiter 96 One Solution reagent and the WTS-1 reagent failed. The formazan crystals formed but remained within the cell structures. Success was achieved using the CellTiter 96 reagent (composed of two solutions - the MTT reagent and a Solubilization solution) which successfully dissolved the formazan crystals into the medium. (Fig. 4.25B)Addition of a 500μg of ascorbic acid gave the best viability. In presence of 1mg of ascorbic acid, viability was half of that at 500μg. Viability in presence of 100μg of ascorbic was even lower. However, addition of EGF to 100μg ascorbic acid brought viability to a level similar to the one displayed in presence of 500μg. Similarly, addition of EGF to 500μg ascorbic acid sightly increased viability. No difference in viability was observed in cultures incubated in presence of low concentrations of ascorbic acid or in its absence (data not shown)

Elaeocyte culture in Schneider medium Elaeocytes grown in Schneider medium remained in culture for over a month. However, cell viability was drastically reduced after 23 days in culture as compared to the viability observed upon plating (Fig. 4.26F). These cells adhered to plastic and glass (Fig. 4.26A& D, E) Some cells were observed

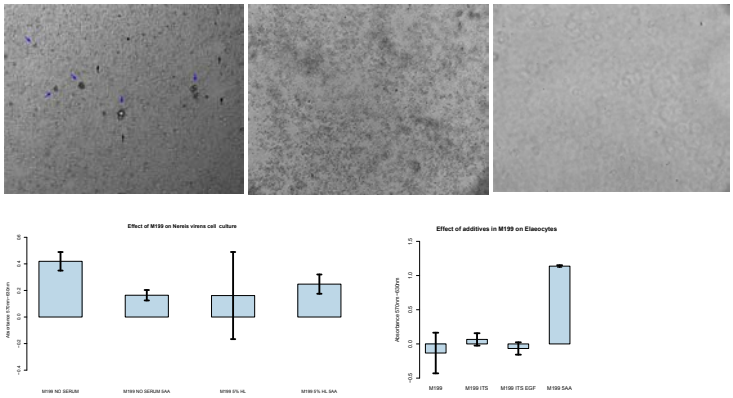


Figure 4.24: Effect of M199 on elaeocyte culture: Top panel result of elaeocyte growth in M199 medium over (from left to right: 3days, 5 days, 2 weeks) Most cells perished (blue arrows indicate surviving elaeocytes) Bottom panel: effect of *Nereis virens* hemolymph supplementation on elaeocyte viability and effect of ITS, EGF and ascorbic acid supplementation on viability as evaluated by the MTT-reduction assay

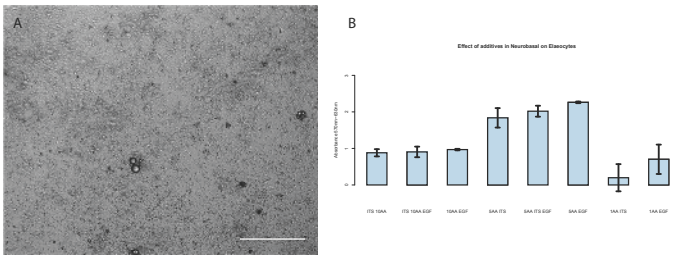


Figure 4.25: Effect of NB and supplementation with ascorbic acid, ITS and EGF on elaeocyte culture (A)Elaeocytes derived from an adult *Nereis virens* after 24hours in culture in Neurobasal medium. Most cells have been replaced with cell debris(B)The effect of supplementation with ascorbic acid

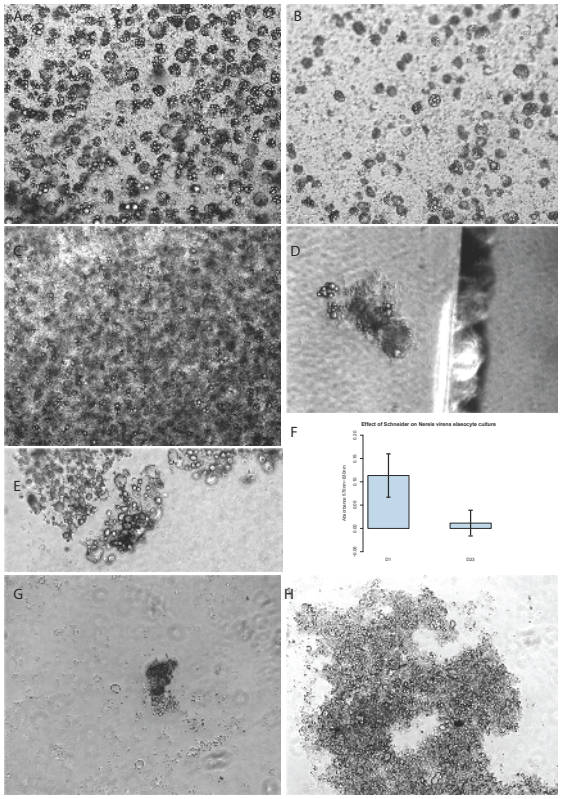


Figure 4.26: Effect of Schneider medium and supplementation with with *Nereis virens* hemolymph . (A)Elaeocytes adhering to plastic in Schneider. (B) Elaeocytes in suspension. (C) Densely plated elaeocytes derived from an adult *Nereis virens* after 24hours in culture in Neurobasal medium. Displayed capacity to both remain in suspension, adhere to plastic. (D) Cells adhered to glass coverslips. (E) Close up (x20) of aggregate of elaeocyte-like cells adhering to glass. (F) Viability of elaeocyte-like cells upon plating compared to viability after 23 days in culture measured by the MTT-reduction assay.(G-H)Elaeocyte-like cells cultured in presence of 5% *Nereis virens* hemolymph. (G)Few cells were observed to adhere to glass. (H)On plastic elaeocytes were observed to adhere to plastic in large aggregates

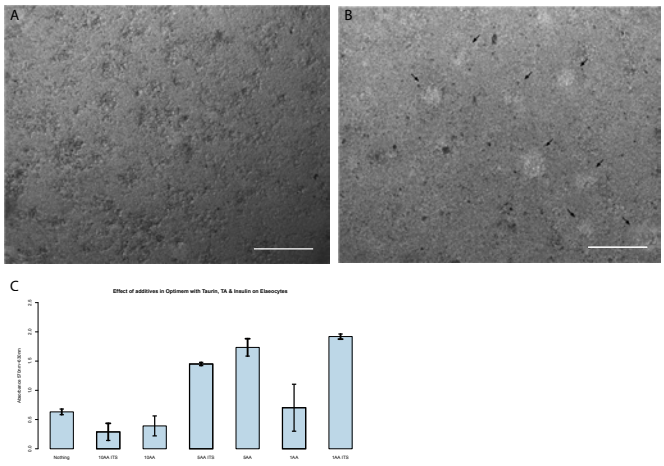


Figure 4.27: Effect of Optimem medium and of Optimem with Taurine, α-tocopherol acetate and insulin supplemented with ascorbic acid and ITS. (A)After 24hours incubation, cells were replaced primarily by cell debris. Both cell debris and remaining cells adhered to plastic (B) Numerous "empty" spaces were observed. (black arrows).(C) Effect of Optimem with Taurine, insulin and α-tochopherol acetate supplementation with ascorbic acid and ITS measured using the MTT-reduction assay.

to remain in suspension (Fig. 4.26B). Supplementation of Schneider medium with 5% *Nereis virens* hemolymph reduced the amount of cells. The cells that remained were clustered in large adhering aggregates (Fig. 4.26G-H).

Elaeocyte culture in Optimem medium Elaeocytes incubated in Optimem rapidly began to die. By day two of culture cells were visibly replaced by cell debris. Furthermore, in close proximity to surviving cells (marked by the presence of large vacuolar structures), "empty" spots were observed. As elaeocytes are capable of phagocytic function, it can be hypothesized that the empty spaces are due to the phagocytosis of the cells that occupied these spaces previously. This was not tested. Optimem supplemented with Taurine, α-tocopherol acetate and insulin showed similar results. Supplementation of Optimem containing Taurine, α-tocopherol acetate and insulin with 500μg ascorbic acid demonstrated the highest viability as measured by the MTT-reduction assay. Supplementation with 1mg and 100μg ascorbic acid reduced cell viability. However, co-supplementation with 100μg ascorbic acid and ITS x1 increased cell viability to the level observed in presence of 500μg ascorbic acid. Supplementation of cells incubated in 500μg ascorbic acid with ITS reduced viability as compared to non-supplemented sample but viability was higher than in absence of ascorbic acid.

Elaeocytes cultured in F12 medium, invariably died. Cells were rapidly replaced with extensive cell debris. All attempts to rescue cells by dilution, change of media or

supplementation with ascorbic acid, ITS, EGF and serum failed (Fig. 4.23).

Culture of tissue explants from *Nereis virens*

Nereis virens used for hemolymph extraction were subsequently dissected and tissue explants were attempted in a medium consisting of 50:50 DMEM: Schneider in presence of 10% FCS in 2 ml in a Nunc 6-well plate or on coverslips in 24well plates. *Nereis virens* has an epidermal nervous system (nervous tissue which closely attached to the epidermis) rendering dissection of nervous tissue alone difficult. These culture attempts derived not from dissociated cells but rather from explants which similarly to explant culture performed with *Platynereis* explants, were let to shed cells into the medium over time, were the most successful(Fig. 4.28). Cells survived for the longest periods of time (Table 4.8). Cells not only left explants but subsequently grew in large elongated clumps (Fig. 4.29 and Fig. 4.30). Curiously, unlike *Platynereis* cell aggregates, *Nereis* cell culture clumps displayed striking autofluorescence at all wavelengths examined, allowing high-level visualization in the absence of staining (Fig. 4.29).

Table 4.8: Survival of *Nereis virens* explants

Tissue dissected	Survival
Epidermis, blood vessel and nervous system	Fixed after 2 days
Epidermis nervous system	Fixed after 2 days
Epidermis muscle	Contamination
Epidermis	Survived 2 months
Parapodia	Survived 2 months
Head	Contamination
Striated muscle	Contamination
Gut	Survived 2 months

The resulting non-contaminated tissue and cells were transferred after 5 days into 50 ml BD Falcon flasks in presence of 20 ml medium (Optimem without serum for gut and parapodia and RPMI with 10% FCS for a mix of *Nereis virens* tissues). *Nereis virens* dissected gut-derived cells were left to grow for 1 month and supplemented with 300 μl insulin. No effect was observed from insulin addition. Attempts to characterize cells resulted in a proliferation of fungus. Fungus was isolated on agar plates supplemented with Guillard's solution (50x). The cultured contamination was identified using barcoding method described above as *Penicillium discolor*.

4.2.13 Adhesion assay on *Nereis virens* cells

As with Platynereis cells, different substrates were tested to determine to which cells adhere best. Results are summarized in table below.

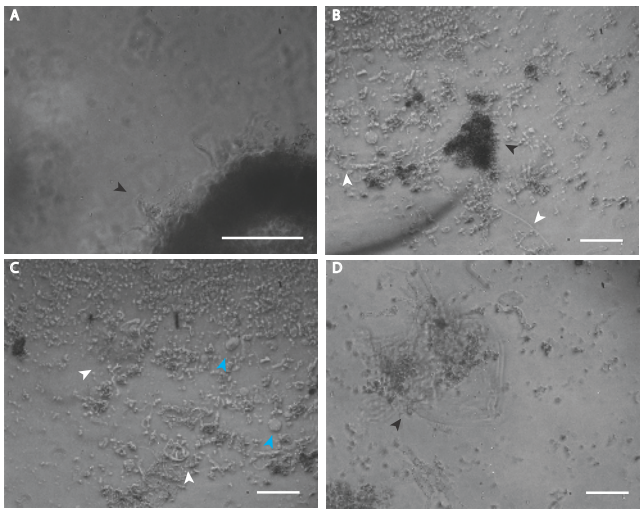


Figure 4.28: Explant culture from dissected adult *Nereis virens*: (A) Parapodial explants in suspension (black arrow indicated muscle-like cells being shed form explant) (B) Small portion that detached from original explant remaining in suspension (black arrow indicating cells being shed from explant) and surrounding muscle-like cells partially adherent to the bottom of cell-bottom well (indicated by white arrows) (C) Muscle-like cells shed from explants (white arrows) and non-muscle-like cells present in culture of unknown origin and nature (blue arrows)(D) Remaining envelope of explant with no cells remaining inside (black arrow)

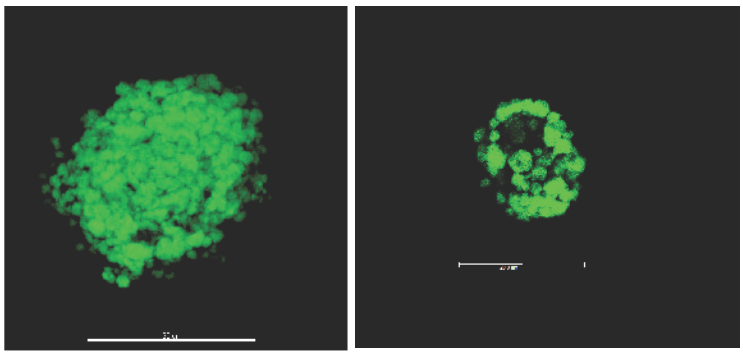


Figure 4.29: Cell culture ”clumps” resulting from *Nereis virens* explant culture displayed considerable autofluorescence, allowing visualization in absence of staining.

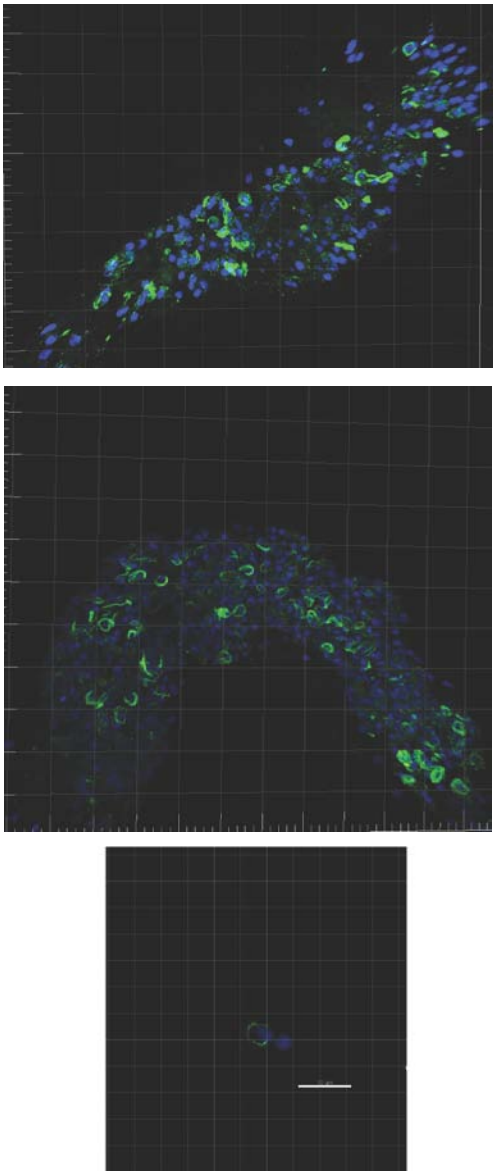


Figure 4.30: Cell culture ”clumps” resulting from *Nereis virens* explant culture, stained with phalloidin (green) and DAPI (blue), bottom panel: a single cell stained with phalloidin and DAPI

Table 4.9: Survival of *Nereis virens* explants

Substrate	Cells adhere	Cells remain in suspension	Staining possibility
Gelatin	N	Y	N
Laminin	N	N	N (cell death)
Poly-L-Lysine	(Y)	Y	Y
Carbon coating	Y	Y	N (carbon dissolves)

4.2.14 Media determination for the of culture of cells derived from *Nematostella*

Numerous attempts have been made to culture Cnidarians in the past by various groups, however with little success. Using the tools developed and tested on *Platynereis* and *Nereis*, attempts were carried out on *Nemtostella*, thanks to its new availability in the lab. Cells were obtained from adult maturing *Nemtostella* and dissociated with Proteinase K at concentrations determined as ideal for *Platynereis* (see Cell dissociation section). Cells were incubated in the commercial media used for *Platynereis* and *Nereis* cell culture attempts with and without the most promising additives (see above). Upon plating cells appeared to be considerably smaller than those obtained from *Platynereis* of *Nereis*. Numerous cells displaying complicated morphologies were observed. Although, most cell culture attempts succumbed to fungal contamination quite rapidly, several survived for weeks and were passed to petri dish after 3 weeks. These cells displayed a different morphology as compared to the cells that were originally plated. COI-based identification of cells was used to confirm that these cells were indeed *Nematostella* cells, however after 2.5 months all cells remaining were identified as *Thraustochytridium*.

4.2.15 Determination of confirmation of origin method

Nematostella contamination identification

Using primers for 18S, ITS and actin proliferating cells were identified as *Thraustochytrium aureum*. *Thraustochytrium aureum* basis of record: Gaertner, Alwin. 1977. Revision of the Thraustochytriaceae (Lower Marine Fungi) I. Ulkenia nov. Gen., with Description of Three New Species. Verüff. Inst. Meeresforch. Bremerh. 16: 139-157

4.3 Discussion

4.3.1 Cell culture limits

Each organism has limits in regard to the environment in which it can survive on the short term or on the long term. *Platynereis* is a isoosmotic marine organism whose cells are sensitive to salt concentrations.

Table 4.10: Survival of *Nematostella* cells

Medium tested	Additive	Time of Survival	
L-15	-	Contaminated in ¡1 week	
L-15x2		-	
DMEM		-	Contaminated in ¡1 week
Neurobasal	-	Contaminated in 2 days	
Optimem		-	Contaminated in 2 days
RPMI	-	Contaminated in ¡1 week	
M199	-	Contaminated in 2 days	
M199 10	%FCS	Contaminated in 3 weeks	
L-15	%FCS	Contaminated in 3 weeks	
L-15x2	%FCS	Contaminated in 3 weeks	
DMEM	%FCS	Contaminated in 3 weeks	
Neurobasal	%FCS	Contaminated in 3 weeks	
Optimem	%FCS	Contaminated in 3 weeks	
RPMI	%FCS	Contaminated in 3 weeks	
M199	%FCS	Contaminated in 3 weeks	
L-15	ITS EGF	Contaminated in 3 weeks	
L-15x2	ITS EGF	Contaminated in 3 weeks	
DMEM	ITS EGF	Contaminated in 3 weeks	
Neurobasal	ITS EGF	Contaminated in 3 weeks	
Optimem	ITS EGF	Contaminated in 3 weeks	
RPMI	ITS EGF	Contaminated in 3 weeks	
M199	ITS EGF	Contaminated in 3 weeks	

4.3.2 Cell culture approaches

Platynereis cell culture was attempted using several approaches. Firstly, much like early XXth century attempts, a minimal medium approach was adopted. Cells were incubated in a medium containing a multitude of undefined components such as sera and *Nereis virens* hemolymph, lyophilized *Nereis virens*, heat treated and macerated adult, larval and unfertilized eggs, chick egg extract and bacterial extract. FCS was observed to be most successful at driving cell metabolism on the long term, however cells did not proliferate. For this reason, a minimal medium approach was considered insufficient for the culture of *Platynereis* cells.

In a parallel effort, attempts were made to take advantage of the numerous commercially available media of known standardized composition. Rich media containing large quantities of energy sources and vitamins were observed to indeed drive cell metabolism and poorer media were observed to slow down cell function. Unlike the minimal medium, cells were observed to invariably go through a drop in cell division and survival after several days in all the commercial media tested.

As a result it was concluded that additional supplements were necessary to find optimal growth conditions. Minimal media conditions were supplemented with a variety

of substances, which for the most part showed a dose dependent negative relationship to cell viability as determined by the MTT-reduction assay. The only substance tested that did not display such a relationship was BMP4. The effect of BMP4 was similar to that of EGF. Due to these discouraging results more efforts were concentrated on the second approach, using defined standardized media supplemented with substances of known composition and concentration.

Based on the success of mussel cell culture by Odintsova and colleagues, particular focus was put on testing media conditions utilized for this organism in *Platynereis* cells. Odintsova and Khomenko achieved successful primary cell differentiation in mussel cells by adding large amounts of glutamate, α-tocopherol acetate, insulin and taurine. As glutamate was observed to be deleterious in high concentrations (inferior to those utilized by Odintsova and Khomenko), the effect of α-tocopherol acetate, insulin and taurine were investigated in parallel with that of the promising candidate from the Minimal Medium screen - EGF. As α-tocopherol acetate is difficult to manipulate at high concentrations due to the necessity to resuspend it in ethanol. Attempts to recycle the amounts of α-tocopherol acetate in culture were made by addition of ascorbic acid. Odintsova carried out her experiments in L15 medium. Therefore, L15 was judged first candidate medium in which to test the different concentrations of each of these additives. As insulin is routinely added to cell culture in the form of ITS (Insulin, Transferrin and selenite salts), this reagent was also tested. Based on qualitative and quantitative assays to determine the effect of these substances, it was observed that neither α-tocopherol acetate, insulin (alone or in conjunction with transferrin and selenite), taurine or EGF acted favorably on *Platynereis* cell culture. Instead, ascorbic acid had a stronger effect on cell maintenance than any of the combinations of the other reagents tested. Secondarily, these substances were tested in media other than L15. Indeed, in the other media tested, cell maintenance and metabolism were observed to be better in presence of ascorbic acid, as compared to all other culture components tested.

4.3.3 Minimal Medium approach

The minimal medium approach was utilized due to lack of information concerning the nutritional and physiological requirements of *Platynereis* cells. Salt concentrations resulting in 820 mOsm/kg to 1100mOsm/kg were judged optimal for dissociated cells of larvae and adults. The only exception to this observation were muscle-like and elaeocyte-like cells. These cells appeared far less sensitive on the short term to salt concentrations. The effect of extreme conditions on these cells was not evaluated precisely. Elaeocyte (macrophage-like cells responsible for phagocytosis of muscle tissue during metamorphosis) are known to possess large acidic vacuoles. Much like muscle cells whose activity is intrinsically dependent on ion fluxes, both these cell types have an intrinsic capacity to control ionic concentrations. pH is closely linked to salt and buffer concentrations. Optimal pH was determined by incubating whole embryos, larva and freshly dissociated cells in an osmotically adjusted solution of NaCl. Unlike most vertebrate cell culture media which are buffered to a pH of 7-7.5, the optimal pH for *Platynereis* cells was determined to be close between 6.5 and 9. Antisceptic and antimycotic requirements were determined. Several types of antimycotic and antibiotic reagents were tested: cell membrane inhibitors, proteins synthesis inhibitors,

β-lactam antibiotics, copper sulfate solution and commercial reagent Unimarine were tested. Due to the lack of characterization of the antibiotic resistance of nereids, several concentrations of several representatives of each class of antibiotic and antimycotic substances were tested: Nystatin, Amphoptericin B, Kanamycin, Streptomycin and Penicillin. Kanamycin was not observed to be able to combat contaminants. Nystatin precipitated in the solution. Amphotptericin B, Streptomycin and Penicillin and Unimarine were observed to both affect contamination and not exert a toxic effect on *Platynereis* cells. Optimal concentrations of each of these reagents were determined. One most also bear in mind that all the antibiotic and antimycotic reagents tested work most efficiently at pH 7 which is in contrast to the optimal pH of the cultured cells. Thus, none of these reagents will function at it peak activity in *Platynereis* cell culture.

4.3.4 Contamination

Contamination of cell culture by microorganisms is a serious problem. As cell are incubated in media rich in nutritive substances that are consumed by contaminants and cultured cells alike. For this reason, removal of contaminants is of utmost importance. As *Platynereis* larvae and adults are not cultured in sterile conditions, obtaining sterile cells is not possible. One method to minimize contaminants was described in Chapter 2. By using density gradient centrifugation, bacteria and cells can be separated. Bacteria remain in the top fraction of the gradient, whereas denser *Platynereis* cells descend in the gradient. This method however, is laborious and does not allow the elimination of protozoan contaminants which bear similar buoyancy to dissociated *Platynereis* cells at the densities tested. Furthermore, fungal contamination was frequently observed. Addition of prophylactic antibiotics and antimycotic is one approach. However, at high concentrations each of antibiotics and antimycotics are toxic to cultured cells. In order to identify contaminants, a method was established to culture contaminants and subsequently perform colony PCR on the contaminants in order to identify them. Most contaminants observed were Penicillium. This is in line with the observation that cultures contaminated with fungi were never observed to also be contaminated with bacteria.

Contamination with fungi was a severe problem and hindrance to further development of *Platynereis* cell culture. Cultures were systematically affected and could not be rescued. For this reason it is of utmost importance to remedy this problem before further attempts are made at cell culture. Rearing source material in a aseptic environment is essential. Spawning parents using UV-treated SFNSW and using gloves at all times diminished the frequency of contaminations. Treating nylon meshes and all tools such as pipette tips with UV for 20 minutes prior to use and proceeding to all manipulations only under a flow hood also diminished the frequency of bacterial contamination. Moreover, more stringent washing steps could be implemented if the amounts of starting material used were higher. If larger batch numbers were used for each trial more material neglected in washing steps allowing more thorough washes. If experiments are to be performed on larvae aged of several days, attempts were made to rear larvae in the antibiotic and antimycotic solutions used for cell culture. Penicillin and Streptomycin are relatively expensive and routine usage of Penicillin and Streptomycin is not affordable. However, when these efforts were made, contamination was not observed

in the cell culture. Ideally, to avoid the presence of other marine organisms, dry food rather than live food should be provided to parents. This is more difficult as unlike live food, dry food rots when it is not consumed and may result in bacterial proliferation. For this reason, additional washes of parents would be necessary. This kind of change in rearing conditions would only be worth implementing a considerable portion of the culture were designated to be used for cell culture attempts.

4.3.5 Perspectives in media component determination

In the present work, several types of methods were tested in order to determine optimal media conditions: using a minimal medium containing ingredients of unknown composition and using defined standardized commercially available media. Using both approaches, it was evident that additional supplementation with further factors is necessary. However, due to frequent contamination and limited starting material, testing a large set of putative media enhancing components was deemed infeasible. A possible solution to this dilemma may be to test several media components simultaneously. One such approach is to make use of Plackett-Burman matrices. This approach was previously successfully utilized Zhao [356] for screening media components for culturing the marine sponge *Hymeniacidon perceive*. The latter correspond to economical experimental designs developed in 1946 by Robin L. Plackett and J. P. Burman, which allows the investigation of n-1 variables in at least n experiments. This design is relies on the frequency of each "level" to be be equal and that each test should be present or absent in equal proportions. The underlying assumption of this kind of set up is that all interactions between the different components tested are insignificant relative to main factor effects. This is a powerful tool which could yield results in screens using low amounts of starting material. Moreover, transfection with expression vectors is a tempting tool. However, for this purpose, high throughput transfection systems need to be established. In the present study, four different methods were tested: electroporation, viral infection, lipofection and plate coating. These methods have been successfully used in other model organisms to transform whole organsms and cells. In my hands, *Platynereis* were not successfully transformed using any of these methods. The difficulty in transfecting marine organisms stems from their inherent need for high salt concentrations which can interfere with electroporation and liposome formation during lipofection.

4.4 Materials and Methods

paragraphAnimal Care Embryos were obtained from an in-house culture of *Platynereis*, maintained by Ms. Diana Bryant as described by [173]. Animals were grown at $18\,^{\circ}\mathrm{C}$, following a light-dark cycle resembling that of the natural moon cycle: approximately one week of artificial moonlight and three weeks of darkness. Mature male and female adults were allowed to spawn and their eggs collected. For young embryo/blastomere dissociation, jelly surrounding the fertilized oocytes was removed after one hour post-fertilization using a 100 microμm-diameter mesh. When dissociating trochophore larvae, larvae were allowed to hatch out of the jelly on their own and the remaining non-

hatched larvae were aspirated using a plastic pasteur pipette. After jelly removal, larvae and embryos were transferred to SFNSW or SFCMFSW.

Trochophore larvae dissociation To obtain cells for primarily culture trochophore larvae were dissociated as described in Chapter 1, using 0.75% Pronase in SFCMFSW on a shaker for 4- 5 hours in a 10mL Falcon tube. The resulting solution was centrifuged at 3500 rpm and the supernatant containing enzyme bacterial contamination and cell debris was removed. Cells were washed using 10mL of SFCMFSW several times. Lack of persisting contamination was verified by resuspending a small amount of cells and observation under a binocular. If cell quality was judged appropriate, cells were plated at a density of 50-100'000 cells/mL. Concentrations were determined using a hemocytometer and volumes were adjusted accordingly. If comparing different media cells were plated in a 10x concentrated stock form and subsequently diluted with 90 μl medium.

Adult dissociation Immature adults were obtained from the in-house culture. Solutions were prepared as for trochophore larva treatment. Worms were placed in an eppendorf. All excess liquid was removed and a pestle was used to grind the animals. Enzyme solutions were added to ground animals and incubated on a shaker on an Eppendorf Thermomixer at 500-750rpm. Alternatively animals were minced using a scalpel or dissection scissors (generously provided by A.Fischer) and incubated with a magnetic stirrer in a 600 mL beaker. Resulting solution was smeared on a 75 microμm cell sorter and the flowthrough examined.

MTT, FDA, NR, TP and total cell number assay 10 μl of MTT reagent (Sigma), or 5 μl of FDA 5mg/ml stock (Sigma), or 0,1% vol. NR, or 0,5% TP were added to 200 μl. in a 96 well. MTT absorption was measured with a luminometer at 450nm and 620nm. The 620nm value was subtracted from the 450nm value. FDA fluorescence was measured using a luminometer at 520nm.

Media Media composition are as indicated in the text and figures.

Electroporation Electroporation was performed as described by Corbo and colleagues. Plasmid was dissolved in 0.8 M mannitol in a total volume of 500μl to a final concentration of at a concentration of 100 μg/ml . Larvae were concentrated in a volume of 200 μl in SFNSW and mixed with the 500 μl of DNA-mannitol solution in an eppendorf. The mixture was transferred to a 0.4cm electroporation cuvette and current pulse was applied as described in the text.

Lipofection Lipofection attempts were carried out as recommended by manufacturer. PCS2+ CMV-GFP was diluted in 50 μl medium to concentrations ranging from 1μg to 0.1μl and mixed gently. Lipofectamine 2000 was mixed before use and added in 2.5 to 0.5μl volumes in a separate 50μl of medium. Solutions were incubated for 5 minutes at room temperature. Lipofectamine 2000 containing solution and DNA containing

solution were combined and incubated for 20 minutes (in a total volume of 100 μl). The full 100μl resulting solution was transferred to a 96 well plate well containing the cells. Plates were gently mixed by rocking and replaced in the incubator.

Transfection by coating attempt Coating of 96 well plates with and without fibronectin and plasmid was performed as routinely used by the ALMF. All necessary solutions were prepared by the ALMF staff. Namely, 0.2% gelatine (0.2g gelatine in 100ml distilled water heated to 56C for 20 min in waterbath for dissolving, filtered with 0.45μm pore filter and cooled down before use and sucrose/ Opti-MEM solution (1.37g sucrose in 10ml Opti-MEM dissolved without shaking on bench). Plasmid gelatin mix was prepared in the following order: 3μl sucrose/Opti-MEM was added to 3.5μl Lipofectamine 2000. The resulting solution was mixed 8 times by pipetting. DNA was added and again mixed 8 times by pipetting. The resulting solution was incubated for 20 minutes at room temperature. In a second step fibronectine was diluted 1:100 in gelatine and 7.25μl solutions were added and mixed 8 times by pipetting. The final volume of the solution was of 18.75μl. Plasmid was prepared in distilled water in a total volume of 800 μl by adding 400 μl water to 16μl plasmid transfection mix and mixed 5 times by pipetting. An addition 400 μl were added. 50 μl diluted plasmid were used per 96 well plate well. Coating was carried out by transferring 50 μl into the respective wells and immediately drying the plates in a speed vacuum for 45 minutes at $50\,^{\circ}\mathrm{C}$, 30 minutes at $49\,^{\circ}\mathrm{C}$- $41\,^{\circ}\mathrm{C}$ and 20 minutes at $37\,^{\circ}\mathrm{C}$. Plates were stored with drying pearls in sealed boxes for future use. For transfection tests, 200, 400 and 800 ng/μl plasmid were tested. PCS2+ CMV-GFP (curtesy the Wittbrodt lab, EMBL Heidelberg) and Ubiquitin-GFP (produced by Dr. Mette Handberg-Thorsager).

Bibliography

[1] E M de Robertis. Evo-devo: variations on ancestral themes. *Cell*, 132(2):185–195, January 2008.

[2] M.J. West-Eberhard. *Developmental plasticity and evolution*. Oxford University Press, USA, 2003.

[3] C. Patterson. Morphological characters and homology, 1982.

[4] S.J. Gould. *The structure of evolutionary theory*. Belknap Press, 2002.

[5] Neil Shubin, Cliff Tabin, and Sean Carroll. Deep homology and the origins of evolutionary novelty. *Nature*, 457(7231):818–823, February 2009.

[6] J. Gerhart, M. Kirschner, and G.T. McVean. Cells, embryos and evolution. *Trends in Biochemical Sciences*, 22(12):495, 1997.

[7] J. Jizba, V. Herout, et al. Polypodine b–a novel ecdysone-like substance from plant material. *Tetrahedron Letters*, 8(51):5139–5143, 1967.

[8] E.M. Deuchar. *Cellular interactions in animal development*. Chapman and Hall, 1975.

[9] W.J. Gehring and K. Ikeo. Pax 6: mastering eye morphogenesis and eye evolution. *Trends in Genetics*, 15(9):371–377, 1999.

[10] W.J. Gehring. The genetic control of eye development and its implications for the evolution of the various eye-types. *International Journal of Developmental Biology*, 46(1):65–74, 2002.

[11] M.F. Land and D.E. Nilsson. *Animal eyes*. Oxford University Press, USA, 2002.

[12] R. Quiring, U. Walldorf, U. Kloter, and W.J. Gehring. Homology of the eyeless gene of drosophila to the small eye gene in mice and aniridia in humans. *Science*, 265(5173):785, 1994.

[13] T. Czerny, G. Halder, U. Kloter, A. Souabni, W.J. Gehring, and M. Busslinger. twin of eyeless, a second pax-6 gene of drosophila, acts upstream of eyeless in the control of eye development. *Molecular cell*, 3(3):297–307, 1999.

[14] I. Hanson and V. Van Heyningen. Pax6: more than meets the eye. *Trends in Genetics*, 11(7):268–272, 1995.

[15] G. Sheng, E. Thouvenot, D. Schmucker, D.S. Wilson, and C. Desplan. Direct regulation of rhodopsin 1 by pax-6/eyeless in drosophila: evidence for a conserved function in photoreceptors. *Genes & development*, 11(9):1122, 1997.

[16] D. Papatsenko, A. Nazina, and C. Desplan. A conserved regulatory element present in all drosophila rhodopsin genes mediates pax6 functions and participates in the fine-tuning of cell-specific expression. *Mechanisms of Development*, 101(1-2):143–153, 2001.

[17] A. Cvekl and J. Piatigorsky. Lens development and crystallin gene expression: many roles for pax-6. *Bioessays*, 18(8):621–630, 1996.

[18] MK Duncan, A. Cvekl, M. Kantorow, and J. Piatigorsky. Development of the ocular lens. robinson ml, lovicu fj., editors, 2004.

[19] Z. Kozmik, M. Daube, E. Frei, B. Norman, L. Kos, L.J. Dishaw, M. Noll, and J. Piatigorsky. Role of pax genes in eye evolution:: A cnidarian paxb gene uniting pax2 and pax6 functions. *Developmental cell*, 5(5):773–785, 2003.

[20] J. Kavaler, W. Fu, H. Duan, M. Noll, and J.W. Posakony. An essential role for the drosophila pax2 homolog in the differentiation of adult sensory organs. *Development*, 126(10):2261, 1999.

[21] W. Fu and M. Noll. The pax2 homolog sparkling is required for development of cone and pigment cells in thedrosophila eye. *Genes & development*, 11(16):2066, 1997.

[22] T. Czerny, M. Bouchard, Z. Kozmik, and M. Busslinger. The characterization of novel pax genes of the sea urchin and drosophila reveal an ancient evolutionary origin of the pax2/5/8 subfamily. *Mechanisms of development*, 67(2):179–192, 1997.

[23] M. Torres, E. Gómez-Pardo, and P. Gruss. Pax2 contributes to inner ear patterning and optic nerve trajectory. *Development*, 122(11):3381, 1996.

[24] W. Fu, H. Duan, E. Frei, and M. Noll. shaven and sparkling are mutations in separate enhancers of the drosophila pax2 homolog. *Development*, 125(15):2943, 1998.

[25] E.T. Dermitzakis and A.G. Clark. Evolution of transcription factor binding sites in mammalian gene regulatory regions: conservation and turnover. *Molecular biology and evolution*, 19(7):1114, 2002.

[26] A.R. Borneman, T.A. Gianoulis, Z.D. Zhang, H. Yu, J. Rozowsky, M.R. Seringhaus, L.Y. Wang, M. Gerstein, and M. Snyder. Divergence of transcription factor binding sites across related yeast species. *Science*, 317(5839):815, 2007.

[27] Dominic Schmidt, Michael D Wilson, Benoit Ballester, Petra C Schwalie, Gordon D Brown, Aileen Marshall, Claudia Kutter, Stephen Watt, Celia P Martinez-Jimenez, Sarah Mackay, Iannis Talianidis, Paul Flicek, and Duncan T Odom. Five-vertebrate ChIP-seq reveals the evolutionary dynamics of transcription factor binding. *Science*, 328(5981):1036–1040, May 2010.

[28] S.J. Gould. *Ontogeny and Phylogeny*. Belknap Harvard Press, 1977.

[29] A.S. Denes, G. Jékely, P.R.H. Steinmetz, F. Raible, H. Snyman, B. Prud'homme, D.E.K. Ferrier, G. Balavoine, and D. Arendt. Molecular architecture of annelid nerve cord supports common origin of nervous system centralization in bilateria. *Cell*, 129(2):277–288, 2007.

[30] S.P. Lv and E. Mayr. On the evolution of photoreceptors and eyes. *Evol. Biol*, 10:207–263, 1977.

[31] R. Tomer, A.S. Denes, K. Tessmar-Raible, and D. Arendt. Profiling by image registration reveals common origin of annelid mushroom bodies and vertebrate pallium. *Cell*, 142(5):800–809, 2010.

[32] D. Arendt and K. Nubler-Jung. Inversion of dorsoventral axis? *Nature*, 371(6492):26, 1994.

[33] R.P. Harvey. Nk-2homeobox genes and heart development. *Developmental biology*, 178(2):203–216, 1996.

[34] J.A. Hoffmann, F.C. Kafatos, C.A. Janeway, and RAB Ezekowitz. Phylogenetic perspectives in innate immunity. *Science*, 284(5418):1313, 1999.

[35] G. Balavoine and A. Adoutte. The segmented urbilateria: a testable scenario. *Integrative and Comparative Biology*, 43(1):137, 2003.

[36] D. Tautz. Segmentation. *Developmental Cell*, 7(3):301–312, 2004.

[37] J.H.L. Hui, F. Raible, N. Korchagina, N. Dray, S. Samain, G. Magdelenat, C. Jubin, B. Segurens, G. Balavoine, D. Arendt, et al. Features of the ancestral bilaterian inferred from platynereis dumerilii parahox genes. *BMC biology*, 7(1):43, 2009.

[38] A. Hejnol and M.Q. Martindale. Acoel development supports a simple planula-like urbilaterian. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 363(1496):1493, 2008.

[39] R.A. Jenner. Evolution of animal body plans: the role of metazoan phylogeny at the interface between pattern and process. *Evolution & Development*, 2(4):208–221, 2000.

[40] C.J. Lowe, M. Wu, A. Salic, L. Evans, E. Lander, N. Stange-Thomann, C.E. Gruber, J. Gerhart, and M. Kirschner. Anteroposterior patterning in hemichordates and the origins of the chordate nervous system. *Cell*, 113(7):853–865, 2003.

[41]

[42] R. Hooke. 1665. *Micrographia. London*.

[43] H. Fleming and R. Haselkorn. Differentiation in nostoc muscorum: nitrogenase is synthesized in heterocysts. *Proceedings of the National Academy of Sciences*, 70(10):2727, 1973.

[44] B.K. Hall. *Keywords and concepts in evolutionary developmental biology*. Discovery Publishing House, 2007.

[45] H.I. Kornblum and D.H. Geschwind. Molecular markers in CNS stem cell research: hitting a moving target. *Nature Reviews Neuroscience*, 2(11):843–846, 2001.

[46] D. Arendt. The evolution of cell types in animals: emerging principles from molecular studies. *Nature Reviews Genetics*, 9(11):868–882, 2008.

[47] Glycosan Glossary. Glycosan glosary@ONLINE, June 2008.

[48] Cell Wikipedia. Wikipedia cell type@ONLINE, June 2008.

[49] T. Bartolomaeus and G. Purschke. *Morphology, molecules, evolution and phylogeny in polychaeta and related taxa*. Springer, 2005.

[50] B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter. Molecular biology of the cell. 2002. *Garland Science*, pages 497–510.

[51] M.J. Rust, M. Bates, and X. Zhuang. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (storm). *Nature methods*, 3(10):793–796, 2006.

[52] E. Betzig, G.H. Patterson, R. Sougrat, O.W. Lindwasser, S. Olenych, J.S. Bonifacino, M.W. Davidson, J. Lippincott-Schwartz, and H.F. Hess. Imaging intracellular fluorescent proteins at nanometer resolution. *Science*, 313(5793):1642, 2006.

[53] A.E.X. Brown, A. Hategan, D. Safer, Y.E. Goldman, and D.E. Discher. Cross-correlated tirf/afm reveals asymmetric distribution of force-generating heads along self-assembled. *Biophysical journal*, 96(5):1952–1960, 2009.

[54] JPS Smith and S. Tyler. The acoel turbellarians: kingpins of metazoan evolution or a specialized offshoot. *The origins and relationships of lower invertebrates. Oxford University Press, Oxford*, pages 123–142, 1985.

[55] RM Rieger, S. Tyler, JPS Smith III, GE Rieger, FW Harrison, and BJ Bogitsh. Platyhelminthes: Turbellaria. *Microscopic anatomy of invertebrates: Volume 3. Platyhelminthes and Nemertinea.*, pages 7–140, 1991.

[56] M. Whitear and EB Lane. Multivillous cells: epidermal sensory cells of unknown function in lamprey skin. *Journal of Zoology*, 201(2):259–272, 1983.

[57] K.H. Pfenninger. Plasma membrane expansion: a neuron's herculean task. *Nat Rev Neurosci*, 10:251–261, 2009.

[58] D. Ferber. Behind the wheel of an expanding axon. *Science*, 299(5611):1307, 2003.

[59] Barry Ritholtz. Your brain is beautiful (and your neurons are particularly attractive). *New York Times*, 30-11:251–261, 2010.

[60] Imaging technology group.

[61] Biodidac - a bank of digital resources for teaching biology.

[62] S.F. Gilbert and S. Sarkar. Embracing complexity: organicism for the 21st century. *Developmental Dynamics*, 219(1):1–9, 2000.

[63] F.J. Mller, L.C. Laurent, D. Kostka, I. Ulitsky, R. Williams, C. Lu, I.H. Park, M.S. Rao, R. Shamir, P.H. Schwartz, et al. Regulatory networks define phenotypic classes of human stem cell lines. *Nature*, 455(7211):401, 2008.

[64] A.S.Â. nchez Alvarado. Regeneration in the metazoans: why does it happen? *Bioessays*, 22:579, 2000.

[65] J. Brockes. Cell differentation: Muscle escapes from a jelly mould. *Current Biology*, 4(11):1030–1032, 1994.

[66] J.E. Jones and J.T. Corwin. Regeneration of sensory cells after laser ablation in the lateral line system: hair cell lineage and macrophage behavior revealed by time-lapse video microscopy. *The Journal of neuroscience: the official journal of the Society for Neuroscience*, 16(2):649, 1996.

[67] M.Q. Martindale and J.Q. Henry. Intracellular fate mapping in a basal metazoan, the ctenophore mnemiopsis leidyi, reveals the origins of mesoderm and the existence of indeterminate cell lineages. *Developmental biology*, 214(2):243–257.

[68] J.Q. Henry, M.Q. Martindale, and B.C. Boyer. The unique developmental program of the acoel flatworm, neochildia fusca. *Developmental Biology*, 220(2):285–295, 2000.

[69] M.K. Vickaryous and B.K. Hall. Human cell type diversity, evolution, development, and classification with special reference to cells derived from the neural crest. *Biological Reviews*, 81(3):425–455, 2006.

[70] G.F. Striedter and R.G. Northcutt. Biological hierarchies and the concept of homology. *Brain, Behavior and Evolution*, 38(4-5):177–189, 1991.

[71] G.A. Wray. Evolutionary dissociations between homologous genes and homologous structures. 1999.

[72] I. Golding, J. Paulsson, S.M. Zawilski, and E.C. Cox. Real-time kinetics of gene activity in individual bacteria. *Cell*, 123(6):1025–1036, 2005.

[73] J.R. Chubb, T. Trcek, S.M. Shenoy, and R.H. Singer. Transcriptional pulsing of a developmental gene. *Current biology*, 16(10):1018–1025, 2006.

[74] A. Raj, C.S. Peskin, D. Tranchina, D.Y. Vargas, and S. Tyagi. Stochastic mrna synthesis in mammalian cells. *PLoS biology*, 4(10):e309, 2006.

[75] A. Raj and A. van Oudenaarden. Nature, nurture, or chance: stochastic gene expression and its consequences, 2008.

[76] D. Zenklusen, D.R. Larson, and R.H. Singer. Single-rna counting reveals alternative modes of gene expression in yeast. *Nature structural & molecular biology*, 15(12):1263–1271, 2008.

[77] R. Losick and C. Desplan. Stochasticity and cell fate. *Science*, 320(5872):65, 2008.

[78] CP Leblond. Classification of cell populations on the basis of their proliferative behavior. *National Cancer Institute Monograph*, 14:119, 1964.

[79] P.F. Langenbruch and W.C. Jones. Body structure of marine sponges. vi. choanocyte chamber structure in the haplosclerida (porifera, demospongiae) and its relevance to the phylogenesis of the group. *Journal of Morphology*, 204(1):1–8, 1990.

[80] J.W. Valentine, A.G. Collins, and C.P. Meyer. Morphological complexity increase in metazoans. *Paleobiology*, 20(2):131–142, 1994.

[81] W. Ponder and D. Lindberg. Phylogeny and evolution of the mollusca. 2008.

[82] D. Arendt, K. Tessmar-Raible, H. Snyman, A.W. Dorresteijn, and J. Wittbrodt. Ciliary photoreceptors with a vertebrate-type opsin in an invertebrate brain. *Science*, 306(5697):869, 2004.

[83] D. Arendt. Evolution of eyes and photoreceptor cell types. *International Journal of Developmental Biology*, 47(7/8):563–572, 2003.

[84] D. Arendt. Genes and homology in nervous system evolution: comparing gene functions, expression patterns, and cell type molecular fingerprints. *Theory in Biosciences*, 124(2):185–197, 2005.

[85] D. Sobral, O. Tassy, and P. Lemaire. Highly divergent gene expression programs can lead to similar chordate larval body plans. *Current Biology*, 19(23):2014–2019, 2009.

[86] E.H. Davidson. *The regulatory genome: gene regulatory networks in development and evolution*. Academic Press, 2006.

[87] J. Roux and M. Robinson-Rechavi. Developmental constraints on vertebrate genome evolution. *PLoS genetics*, 4(12):e1000311, 2008.

[88] B. Prud'Homme and N. Gompel. Evolutionary biology: Genomic hourglass. *Nature*, 468(7325):768–769, 2010.

[89] A.T. Kalinka, K.M. Varga, D.T. Gerrard, S. Preibisch, D.L. Corcoran, J. Jarrells, U. Ohler, C.M. Bergman, and P. Tomancak. Gene expression divergence recapitulates the developmental hourglass model. *Nature*, 468(7325):811–814, 2010.

[90] D. Whitmore, N.S. Foulkes, and P. Sassone-Corsi. Light acts directly on organs and cells in culture to set the vertebrate circadian clock. *Nature*, 404(6773):87–91, 2000.

[91] A.J.F. Carr and D. Whitmore. Imaging of single light-responsive clock cells reveals fluctuating free-running periods. *Nature cell biology*, 7(3):319–321, 2005.

[92] N.B. Haas, C.A. Cantwell, P.F. Johnson, and JB Burch. Dna-binding specificity of the par basic leucine zipper protein vbp partially overlaps those of the c/ebp and creb/atf families and is influenced by domains that flank the core basic region. *Molecular and cellular biology*, 15(4):1923, 1995.

[93] F. Wollnik, W. Brysch, E. Uhlmann, F. Gillardon, R. Bravo, M. Zimmermann, KH Schlingensiepen, and T. Herdegen. Block of c-fos and junb expression by antisense oligonucleotides inhibits light-induced-phase shifts of the mammalian circadian clock. *European Journal of Neuroscience*, 7(3):388–393, 1995.

[94] F. Gachon, F.F. Olela, O. Schaad, P. Descombes, and U. Schibler. The circadian par-domain basic leucine zipper transcription factors dbp, tef, and hlf modulate basal and inducible xenobiotic detoxification. *Cell Metabolism*, 4(1):25–36, 2006.

[95] K. Obrietan, S. Impey, D. Smith, J. Athos, and D.R. Storm. Circadian regulation of camp response element-mediated gene expression in the suprachiasmatic nuclei. *Journal of Biological Chemistry*, 274(25):17748, 1999.

[96] B.D. Weger, M. Sahinbas, G.W. Otto, P. Mracek, O. Armant, D. Dolle, K. Lahiri, D. Vallone, L. Ettwiller, R. Geisler, et al. The Light Responsive Transcriptome of the Zebrafish: Function and Regulation. *PloS one*, 6(2):e17080, 2011.

[97] Z. Ben-Moshe, G. Vatine, S. Alon, A. Tovin, P. Mracek, N.S. Foulkes, and Y. Gothilf. Multiple par and e4bp4 bzip transcription factors in zebrafish: Diverse spatial and temporal expression patterns. *Chronobiology International*, 27(8):1509–1531, 2010.

[98] E.S. KAWASAKI. Microarrays and the gene expression profile of a single cell. *Annals of the New York Academy of Sciences*, 1020(1):92–100, 2004.

[99] J. Hipp, J. Cheng, JC Hanson, W. Yan, P. Taylor, N. Hu, J. Rodriguez-Canales, MA Tangrea, MR Emmert-Buck, and U. Balis. Sivq-aided laser capture microdissection: A tool for high-throughput expression profiling. *Journal of Pathology Informatics*, 2(1):19, 2011.

[100] K.E. Aaltonen, A. Ebbesson, C. Wigerup, and I. Hedenfalk. Laser capture microdissection(lcm) and whole genome amplification(wga) of dna from normal breast tissue- optimization for genome wide array analyses. *BMC Research Notes*, 4(1):69, 2011.

[101] Worthington Biochemical. Worthington tissue dissociation guide@ONLINE, June 2009.

[102] B. Rinkevich, M. Ilan, and R. Blisko. Further steps in the initiation of cell cultures from embryos and adult sponge colonies. *In Vitro Cellular & Developmental Biology-Animal*, 34(10):753–756, 1998.

[103] V. Schmid, R. Stidwill, A. Bally, B. Marcum, and P. Tardent. Heat dissociation and maceration of marine Cnidaria. *Development Genes and Evolution*, 190(3).

[104] R.D. Gates, G. Baghdasarian, and L. Muscatine. Temperature stress causes host cell detachment in symbiotic cnidarians: implications for coral bleaching. *The Biological Bulletin*, 182(3):324, 1992.

[105] M.K. Khalesi. Cell cultures from the symbiotic soft coral Sinularia flexibilis. *In Vitro Cellular & Developmental Biology-Animal*, 44(8):330–338, 2008.

[106] T. Itami, M. Maeda, M. Kondo, and Y. Takahashi. Primary culture of lymphoid organ cells and haemocytes of kuruma shrimp, penaeus japonicus. *Methods in cell science*, 21(4):237–244, 1999.

[107] C.W. Dunn, A. Hejnol, D.Q. Matus, K. Pang, W.E. Browne, S.A. Smith, E. Seaver, G.W. Rouse, M. Obst, G.D. Edgecombe, et al. Broad phylogenomic sampling improves resolution of the animal tree of life. *Nature*, 452(7188):745–749, 2008.

[108] D. Sipkema, R. Van Wielink, AAM Van Lammeren, J. Tramper, R. Osinga, and RH Wijffels. Primmorphs from seven marine sponges: formation and structure. *Journal of biotechnology*, 100(2):127–139, 2003.

[109] M.C. McKay and P.A.V. Anderson. Preparation and properties of cnidocytes from the sea anemone Anthopleura elegantissima. *The Biological Bulletin*, 174(1):47, 1988.

[110] R. Willoughby and S.A. Pomponi. Quantitative assessment of marine sponge cells in vitro: development of improved growth medium. *In Vitro Cellular & Developmental Biology-Animal*, 36(3):194–200, 2000.

[111] U. Frank, C. Rabinowitz, and B. Rinkevich. In vitro establishment of continuous cell cultures and cell lines from ten colonial cnidarians. *Marine Biology*, 120(3):491–499, 1994.

[112] I.J. Domart-Coulon, D.C. Elbert, E.P. Scully, P.S. Calimlim, and G.K. Ostrander. Aragonite crystallization in primary cell cultures of multicellular isolates from a hard coral, Pocillopora damicornis. *Proceedings of the National Academy of Sciences of the United States of America*, 98(21):11885, 2001.

[113] T.J. Fan and X.F. Wang. In vitro culture of embryonic cells from the shrimp, Penaeus chinensis. *Journal of experimental marine biology and ecology*, 267(2):175–184, 2002.

[114] LM Tapay, Y. Lu, RB Gose, JA Brock, and PC Loh. Infection of Yellow-Head Virus (YHV) and Chinese Baculo-like Virus (CBV) in Two Species of Penaeid Shrimp, Penaeus stylirostris (Stimpson) and P. vannamei (Boone). *World Aquaculture, Bangkok, Thailand*, 1996.

[115] L. Owens and J. Smith. Early attempts at production of prawn cell lines. *Methods in cell science*, 21(4):207–212, 1999.

[116] W. Assavalapsakul, D.R. Smith, and S. Panyim. Propagation of infectious yellow head virus particles prior to cytopathic effect in primary lymphoid cell cultures of penaeus monodon. *Diseases of aquatic organisms*, 55(3):253–258, 2003.

[117] H. Shike, C. Shimizu, KS Klimpel, and JC Burns. Expression of foreign genes in primary cultured cells of the blue shrimp penaeus stylirostris. *Marine Biology*, 137(4):605–611, 2000.

[118] A. UMA, TG PRABHAKAR, A. KOTEESWARAN, and G. RAVIKUMAR. Establishment of primary cell culture from hepatopancreas of penaeus monodon for the study of white spot syndrome virus (wssv) popular. 2002.

[119] CA Fraser and MR Hall. Studies on primary cell cultures derived from ovarian tissue of penaeus monodon. *Methods in cell science*, 21(4):213–218, 1999.

[120] AL Mulford, F. Lyng, C. Mothersill, and B. Austin. Development and characterization of primary cell cultures from the hematopoietic tissues of the dublin bay prawn, nephrops norvegicus. *Methods in cell science*, 22(4):265–275, 2000.

[121] W.N. Wang, H. Liang, A.L. Wang, T. Chen, S.E. Zhang, and R.Y. Sun. Effect of ph and zn2+ on subcultured muscle cells from macrobrachium nipponense. *Methods in cell science*, 22(4):277–284, 2000.

[122] K. Okano, K. Shimizu, C.G. Satuito, and N. Fusetani. Enzymatic isolation and culture of cement secreting cells from cypris larvae of the barnacle Megabalanus rosa. *Biofouling*, 12(1):149–159, 1998.

[123] AL Mulford and B. Austin. Development of primary cell cultures from nephrops norvegicus. *Methods in cell science*, 19(4):269–275, 1998.

[124] C. Moss, P.W. Beesley, M.C. Thorndyke, and T. Bollner. Preliminary observations on ascidian and echinoderm neurons and neural explants in vitro. *Tissue and Cell*, 30(5):517–524, 1998.

[125] I. Domart-Coulon, D. Doumenc, S. Auzoux-Bordenave, and Y. Fichant. Identification of media supplements that improve the viability of primarily cell cultures ofcrassostrea gigas oysters. *Cytotechnology*, 16(2):109–120, 1994.

[126] S. Koyama and M. Aizawa. Tissue culture of the deep-sea bivalve calyptogena soyoae. *Extremophiles*, 4(6):385–389, 2000.

[127] F. Le Marrec-Croq, D. Glaise, C. Guguen-Guillouzo, C. Chesne, A. Guillouzo, V. Boulo, and G. Dorange. Primary cultures of heart cells from the scallp pecten maximus (mollusca-bivalvia). *In Vitro Cellular & Developmental Biology-Animal*, 35(5):289–295, 1999.

[128] T. Renault, G. Flaujac, and R.M. Le Deuff. Isolation and culture of heart cells from the european flat oyster, ostrea edulis. *Methods in cell science*, 17(3):199–205, 1995.

[129] J.T. Buchanan, J.F. La Peyre, R.K. Cooper, and T.R. Tiersch. Improved attachment and spreading in primary cell cultures of the eastern oyster, crassostrea virginica. *In Vitro Cellular & Developmental Biology-Animal*, 35(10):593–598, 1999.

[130] G. Le Pennec and M. Le Pennec. Acinar primary cell culture from the digestive gland of pecten maximus (l.): an original model for ecotoxicological purposes. *Journal of experimental marine biology and ecology*, 259(2):171–187, 2001.

[131] SN Chen and CS Wang. Establishment of cell lines derived from oyster, crassostrea gigas thunberg and hard clam, meretrix lusoria r
”oding. *Methods in cell science*, 21(4):183–192, 1999.

[132] C. Birmelin, RK Pipe, PS Goldfarb, and DR Livingstone. Primary cell-culture of the digestive gland of the marine mussel mytilus edulis: a time-course study of antioxidant-and biotransformation-enzyme activity and ultrastructural changes. *Marine Biology*, 135(1):65–75, 1999.

[133] NA Odintsova, SV Plotnikov, and AA Karpenko. Isolation and partial characterization of myogenic cells from mussel larvae in vitro. *Tissue and Cell*, 32(5):417–424, 2000.

[134] H. Hanana, H. Talarmin, JP Pennec, M. Droguet, E. Gobin, P. Marcorelle, and G. Dorange. Establishment of functional primary cultures of heart cells from the clam ruditapes decussatus. *Cytotechnology*, pages 1–11, 2011.

[135] G. Lang, N. Nomura, and M. Matsumura. Growth by cell division in shrimp (penaeus japonicus) cell culture. *Aquaculture*, 213(1-4):73–83, 2002.

[136] S. Ikegami, TS Okada, and SS Koide. ON THE ROLE OF CALCIUM IONS IN OOCYTE MATURATION IN THE POLYCHAETE CHAETOPTERUS PERGAMENTACEUS*. *Development, Growth & Differentiation*, 18(1):33–43, 1976.

[137] W. Westheide and R.M. Rieger. Cuticle ultrastructure of hesionid polychaetes (Annelida). *Zoomorphology*, 91(1):1–18, 1978.

[138] PL Holborow, MS Laverack, and VC Barber. Cilia and other surface structures of the trochophore of Harmothoe imbricata (Polychaeta). *Cell and Tissue Research*, 98(2):246–261, 1969.

[139] W. Heimler. *Untersuchungen zur Larvalentwicklung von Lanice conchilega (Pallas) 1766 [siebzehnhundertsechsundsechzig](Polychaeta, Terebellomorpha).* PhD thesis, 1981.

[140] W. HEIMLER. Untersuchungen zur Larvalentwicklung von Lanice conchilega (PALLAS) 1766 (Polychaeta/Terebellomorpha). III: Bau und Struktur der Aulophora-Larve= Sur le développement larvaire de Lanice conchilega (Pallas) 1766 (Polychaeta, Terebellomorpha). III Organisation et structure de la larve aulophoreOn the larval development of Lanice conchilega (Pallas) 1766 (Polychaeta, Terebellomorpha) III Organization and structure of the aulophora-larva. *Zoologische Jahrb*
"ucher. Abteilung f
"ur Anatomie und Ontogenie der Tiere, 110(4):411–478, 1983.

[141] U. Schl
"otzer-Schrehardt. *Ultrastrukturelle Untersuchungen zur Reproduktion und Postembryonalentwicklung einschliesslich Adultorganisation von Pygospio elegans Claparède, 1863 (Polychaeta, Spionidae).* PhD thesis, Erlangen., 1992.

[142] M.B. Rotman. Cytotoxicity assays in cell culturing devices, December 17 1985. US Patent 4,559,299.

[143] J. Carmichael, W.G. DeGraff, A.F. Gazdar, J.D. Minna, and J.B. Mitchell. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Research*, 47(4):936, 1987.

[144] W. Strober. Trypan blue exclusion test of cell viability. 2001.

[145] L. Black and MC Berenbaum. Factors affecting the dye exclusion test for cell viability. *Experimental cell research*, 35(1):9–13, 1964.

[146] H. Babich, JA Puerner, and E. Borenfreund. In vitro cytotoxicity of metals to bluegill (bf-2) cells. *Archives of environmental contamination and toxicology*, 15(1):31–37, 1986.

[147] E.B. Wilson. On cleavage and mosaic-work. *Development Genes and Evolution*, 3(1):19–26, 1896.

[148] D.P. Costello. The relations of the plasma membrane, vitelline membrane, and jelly in the egg of Nereis limbata. *The Journal of General Physiology*, 32(3):351, 1949.

[149] F. Moser. Studies on a cortical layer response to stimulating agents in the Arbacia egg. I. Response to insemination. *Journal of Experimental Zoology*, 80(3):423–445, 1939.

[150] R.S. Lillie. ON THE EFFECTS OF VARIOUS SOLUTIONS ON CILIARY AND MUSCULAR MOVEMENT IN THE LARVÆ OF ARENICOLA AND POLYGORDIUS. II. *American Journal of Physiology–Legacy Content*, 7(1):25, 1902.

[151] C. Henley. Exaggerated elevation of the fertilization membrane of Chaetopterus eggs, resulting from cold-treatment. *The Biological Bulletin*, 117(2):284, 1959.

[152] A.B. Novikoff. Embryonic Determination in the Annelid, Sabellaria vulgaris I. The Differentiation of Ectoderm and Endoderm When Separated through Induced Exogastrulation. *Biological Bulletin*, 74(2):198–210, 1938.

[153] A.B. Novikoff. Embryonic determination in the annelid, Sabellaria vulgaris: II. Transplantation of polar lobes and blastomeres as a test of their inducing capacities. *The Biological Bulletin*, 74(2):211, 1938.

[154] DP Costello. Membrane removal from the egg of the annelid, Hydroides. *Biol. Bull*, 115:349, 1958.

[155] R. Lovelace. The effects of precocious sperm entry on the egg of Nereis limbata. *Journal of Experimental Zoology*, 112(1):79–107, 1949.

[156] A.B. NOVIKOFF. Changes at the surface of Nereis limbata eggs after insemination. *Journal of Experimental Biology*, 16(4):403, 1939.

[157] K. Muneoka. A new method for mechanical dechorionation of ascidian eggs* 1. *Developmental Biology*, 74(2):486–489, 1980.

[158] A.W.C. Dorresteijn. Quantitative analysis of cellular differentiation during early embryogenesis ofPlatynereis dumerilii. *Development Genes and Evolution*, 199(1):14–30, 1990.

[159] A.W.C. Dorresteijn and B. Kluge. *On the establishment of polarity in polychaete eggs*. New York: Plenum, 1990.

[160] L. Silverman and D. Glick. The reactivity and staining of tissue proteins with phosphotungstic acid. *The Journal of cell biology*, 40(3):761, 1969.

[161] V.J. Pileggi, J. Di Giorgio, and D.K. Wybenga. A one-tube serum uric acid method using phosphotungstic acid as protein precipitant and color reagent. *Clinica Chimica Acta*, 37:141–149, 1972.

[162] G. Quintarelli, R. Zito, and JA Cifonelli. On phosphotungstic acid staining. i. *Journal of Histochemistry & Cytochemistry*, 19(11):641, 1971.

[163] AE Heacox, A. Fischer, and H.R. Frangenberg. Development of a medium for in vitro culture of oocytes from the polychaete nereis virens. *In Vitro Cellular & Developmental Biology-Plant*, 19(11):825–832, 1983.

[164] N. Devauchelle, G. Dorange, and C. Faure. A technique for separating high-and low-quality embryos of the scallop, Pecten maximus L. *Aquaculture*, 120(3-4):341–346, 1994.

[165] N.A. Odintsova, V.A. Dyachuk, and L.P. Nezlin. Muscle and neuronal differentiation in primary cell culture of larval Mytilus trossulus (Mollusca: Bivalvia). *Cell and Tissue Research*, 339(3):625–637, 2010.

[166] H. Kang and A.S. Alvarado. Flow cytometry methods for the study of cell-cycle parameters of planarian stem cells. *Developmental Dynamics*, 238(5):1111–1117, 2009.

[167] K. Kurimoto, Y. Yabuta, Y. Ohinata, and M. Saitou. Global single-cell cdna amplification to provide a template for representative high-density oligonucleotide microarray analysis. *Nature protocols*, 2(3):739–752, 2007.

[168] D.J. Gusseck and J.L. Hedrick. A molecular approach to fertilization* 1:: I. disulfide bonds in xenopus laevis jelly coat and a molecular hypothesis for fertilization. *Developmental Biology*, 25(3):337–347, 1971.

[169] A.W.C. Dorresteijn, H. Bornewasser, and A. Fischer. A correlative study of experimentally changed first cleavage and janus development in the trunk of platynereis dumerilii (annelida, polychaeta). *Development Genes and Evolution*, 196(1):51–58, 1987.

[170] A.W.C. Dorresteijn and P. Eich. Experimental change of cytoplasmic composition can convert determination of blastomeres inplatynereis dumerilii (annelida, polychaeta). *Development Genes and Evolution*, 200(6):342–351, 1991.

[171] C. Ackermann, A. Dorresteijn, and A. Fischer. Clonal domains in post-larval platynereis dumerilii (annelida: Polychaeta). *Journal of morphology*, 266(3):258–280, 2005.

[172] S.Q. Schneider and B. Bowerman. [beta]-catenin asymmetries after all animal/vegetal-oriented cell divisions in platynereis dumerilii embryos mediate binary cell-fate specification. *Developmental cell*, 13(1):73–86, 2007.

[173] A.W.C. Dorresteijn, B. O'Grady, A. Fischer, E. Porchet-Henneré, and Y. Boilly-Marer. Molecular specification of cell lines in the embryo of platynereis (annelida). *Development Genes and Evolution*, 202(5):260–269, 1993.

[174] G. Coppola and D.H. Geschwind. Microarrays and the microscope: balancing throughput with resolution. *The Journal of Physiology*, 575(2):353–359, 2006.

[175] N.Q. Balaban, J. Merrin, R. Chait, L. Kowalik, and S. Leibler. Bacterial persistence as a phenotypic switch. *Science*, 305(5690):1622, 2004.

[176] S.J. Altschuler and L.F. Wu. Cellular Heterogeneity: Do Differences Make a Difference? *Cell*, 141(4):559–563, 2010.

[177] A. Wilson, E. Laurenti, G. Oser, R.C. van der Wath, W. Blanco-Bose, M. Jaworski, S. Offner, C.F. Dunant, L. Eshkind, E. Bockamp, et al. Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. *Cell*, 135(6):1118–1129, 2008.

[178] J.E. Ferrell and E.M. Machleder. The biochemical basis of an all-or-none cell fate switch in xenopus oocytes. *Science*, 280(5365):895, 1998.

[179] G.H. Heppner. Tumor heterogeneity. *Cancer research*, 44(6):2259, 1984.

[180] H. Rubin. The significance of biological heterogeneity. *Cancer and Metastasis Reviews*, 9(1):1–20, 1990.

[181] K.E. Gascoigne and S.S. Taylor. Cancer cells display profound intra-and interline variation following prolonged exposure to antimitotic drugs. *Cancer Cell*, 14(2):111–122, 2008.

[182] K.A. Janes, C.C. Wang, K.J. Holmberg, K. Cabral, and J.S. Brugge. Identifying single-cell molecular programs by stochastic profiling. *Nature methods*, 7(4):311, 2010.

[183] Luke P. Lee and Dino Di Carlo. *Single Cell Analysis for Quantitative Systems Biology*, pages 135–160. Wiley-VCH Verlag GmbH & Co. KGaA, 2009.

[184] G. Brady. Expression profiling of single mammalian cells-small is beautiful. *Yeast*, 17(3):211–217, 2000.

[185] R. Todd and D.H. Margolin. Challenges of single-cell diagnostics: analysis of gene expression. *Trends in Molecular Medicine*, 8(6):254–257, 2002.

[186] N. Tremain, J. Korkko, D. Ibberson, G.C. Kopen, C. DiGirolamo, and D.G. Phinney. Microsage analysis of 2,353 expressed genes in a single cell-derived colony of undifferentiated human mesenchymal stem cells reveals mrnas of multiple cell lineages. *Stem Cells*, 19(5):408–418, 2001.

[187] V. Nygaard and E. Hovig. Options available for profiling small samples: a review of sample amplification technology when combined with microarray profiling. *Nucleic acids research*, 34(3):996, 2006.

[188] A.K. Dixon, P.J. Richardson, K. Lee, N.P. Carter, and T.C. Freeman. Expression profiling of single cells using 3 prime end amplification (tpea) pcr. *Nucleic Acids Research*, 26(19):4426, 1998.

[189] T. Miyamoto, H. Iwasaki, B. Reizis, M. Ye, T. Graf, I.L. Weissman, and K. Akashi. Myeloid or lymphoid promiscuity as a critical step in hematopoietic lineage commitment. *Developmental cell*, 3(1):137–147, 2002.

[190] J.S. Marcus, W.F. Anderson, and R. Stephen. Microfluidic single-cell mrna isolation and analysis. *Analytical chemistry*, 78(9):3084–3089, 2006.

[191] S. Wang, D. Irimia, A. Jayaraman, M. Toner, and M.L. Yarmush. A high-throughput microfluidic real-time gene expression living cell array. *Lab on a Chip*, 7(1):77–85, 2007.

[192] P.A. Auroux, Y. Koc, A. Manz, and PJR Day. Miniaturised nucleic acid analysis. *Lab on a Chip*, 4(6):534–546, 2004.

[193] C. Zhang, J. Xu, W. Ma, and W. Zheng. Pcr microfluidic devices for dna amplification. *Biotechnology advances*, 24(3):243–284, 2006.

[194] A.Y. Fu, C. Spence, A. Scherer, F.H. Arnold, and S.R. Quake. A microfabricated fluorescence-activated cell sorter. *Nature biotechnology*, 17(11):1109–1111, 1999.

[195] M. Hashimoto, P.C. Chen, M.W. Mitchell, D.E. Nikitopoulos, S.A. Soper, and M.C. Murphy. Rapid pcr in a continuous flow device. *Lab Chip*, 4(6):638–645, 2004.

[196] O. Kalinina, I. Lebedeva, J. Brown, and J. Silver. Nanoliter scale pcr with taqman detection. *Nucleic acids research*, 25(10):1999, 1997.

[197] J. Liu, M. Enzelberger, and S. Quake. A nanoliter rotary device for polymerase chain reaction. *Electrophoresis*, 23(10):1531–1536, 2002.

[198] P.J. Obeid, T.K. Christopoulos, H.J. Crabtree, and C.J. Backhouse. Microfabricated device for dna and rna amplification by continuous-flow polymerase chain reaction and reverse transcription-polymerase chain reaction with cycle number selection. *Analytical chemistry*, 75(2):288–295, 2003.

[199] T. Thorsen, S.J. Maerkl, and S.R. Quake. Microfluidic large-scale integration. *Science*, 298(5593):580, 2002.

[200] L.C. Waters, S.C. Jacobson, N. Kroutchinina, J. Khandurina, R.S. Foote, and J.M. Ramsey. Microchip device for cell lysis, multiplex pcr amplification, and electrophoretic sizing. *Analytical chemistry*, 70(1):158–162, 1998.

[201] A. Khademhosseini, R. Langer, J. Borenstein, and J.P. Vacanti. Microscale technologies for tissue engineering and biology. *Proceedings of the National Academy of Sciences of the United States of America*, 103(8):2480, 2006.

[202] C. Hartshorn, A. Anshelevich, and L.J. Wangh. Rapid, single-tube method for quantitative preparation and analysis of RNA and DNA in samples as small as one cell. *BMC biotechnology*, 5(1):2, 2005.

[203] P.K. Jena, A.H. Liu, D.S. Smith, and L.J. Wysocki. Amplification of genes, single transcripts and cdna libraries from one cell and direct sequence analysis of amplified products derived from one molecule. *Journal of immunological methods*, 190(2):199–213, 1996.

[204] Y. Zhang, C. Ma, T. Delohery, B. Nasipak, B.C. Foat, A. Bounoutas, H.J. Bussemaker, S.K. Kim, and M. Chalfie. Identification of genes expressed in c. elegans touch receptor neurons. *Nature*, 418(6895):331–335, 2002.

[205] M.E. Colosimo, A. Brown, S. Mukhopadhyay, C. Gabel, A.E. Lanjuin, A.D.T. Samuel, and P. Sengupta. Identification of thermosensory and olfactory neuron-specific genes via expression profiling of single neuron types. *Current biology*, 14(24):2245–2251, 2004.

[206] R.M. Fox, S.E. Von Stetina, S.J. Barlow, C. Shaffer, K.L. Olszewski, J.H. Moore, D. Dupuy, M. Vidal, and D.M. Miller. A gene expression fingerprint of c. elegans embryonic motor neurons. *BMC genomics*, 6(1):42, 2005.

[207] J. Buchstaller, L. Sommer, M. Bodmer, R. Hoffmann, U. Suter, and N. Mantei. Efficient isolation and gene expression profiling of small numbers of neural crest stem cells and developing schwann cells. *The journal of neuroscience*, 24(10):2357, 2004.

[208] P. Arlotta, B.J. Molyneaux, J. Chen, J. Inoue, R. Kominami, and J.D. Macklis. Neuronal subtype-specific genes that control corticospinal motor neuron development in vivo. *Neuron*, 45(2):207–221, 2005.

[209] K. Sugino, C.M. Hempel, M.N. Miller, A.M. Hattox, P. Shapiro, C. Wu, Z.J. Huang, and S.B. Nelson. Molecular taxonomy of major neuronal classes in the adult mouse forebrain. *Nature neuroscience*, 9(1):99–107, 2005.

[210] P.G. Marciano, J. Brettschneider, E. Manduchi, J.E. Davis, S. Eastman, R. Raghupathi, K.E. Saatman, T.P. Speed, C.J. Stoeckert, J.H. Eberwine, et al. Neuron-specific mrna complexity responses during hippocampal apoptosis after traumatic brain injury. *The Journal of neuroscience*, 24(12):2866, 2004.

[211] H. Kunitomo, H. Uesugi, Y. Kohara, and Y. Iino. Identification of ciliated sensory neuron-expressed genes in caenorhabditis elegans using targeted pull-down of poly (a) tails. *Genome Biology*, 6(2):R17, 2005.

[212] Z. Yang, H.J. Edenberg, and R.L. Davis. Isolation of mrna from specific tissues of drosophila by mrna tagging. *Nucleic Acids Research*, 33(17):e148, 2005.

[213] M.G. Carter, A.A. Sharov, V. VanBuren, D.B. Dudekula, C.E. Carmack, C. Nelson, and M.S.H. Ko. Transcript copy number estimation using a mouse whole-genome oligonucleotide microarray. *Genome biology*, 6(7):R61, 2005.

[214] L. Zhang, W. Zhou, V.E. Velculescu, S.E. Kern, R.H. Hruban, S.R. Hamilton, B. Vogelstein, and K.W. Kinzler. Gene expression profiles in normal and cancer cells. *Science*, 276(5316):1268, 1997.

[215] V.E. Velculescu, S.L. Madden, L. Zhang, A.E. Lash, J. Yu, C. Rago, A. Lal, C.J. Wang, G.A. Beaudry, K.M. Ciriello, et al. Analysis of human transcriptomes. *Nature Genetics*, 23(4):387–388, 1999.

[216] M. Mahadevappa and J.A. Warrington. A high-density probe array sample preparation method using 10-to 100-fold fewer cells. *Nature biotechnology*, 17(11):1134, 1999.

[217] D. Baltimore. Rna-dependent dna polymerase¡ n virions of rous sarcoma virus. *Nature*, 226(121):1, 1970.

[218] HM Temin and S. Mizutani. Rna-dependent dna polymerase in virions of rna tumour viruses. *Nature*, 226:1211, 1970.

[219] DL Kacian, S. Spiegelman, A. Bank, M. Terada, S. Metafora, L. Dow, and PA Marks. In vitro synthesis of dna components of human genes for globins. *Nature*, 235(58):167–169, 1972.

[220] J. Ross, H. Aviv, E. Scolnick, and P. Leder. In vitro synthesis of dna complementary to purified rabbit globin mrna. *Proceedings of the National Academy of Sciences of the United States of America*, 69(1):264, 1972.

[221] I.M. VERMA, G.F. TEMPLE, H. FAN, and D. BALTIMORE. In vitro synthesis of dna complementary to rabbit reticulocyte 10s rna. *Nature*, 235(58):163–167, 1972.

[222] S.N. Cohen, A.C.Y. Chang, H.W. Boyer, and R.B. Helling. Construction of biologically functional bacterial plasmids in vitro. *Proceedings of the National Academy of Sciences of the United States of America*, 70(11):3240, 1973.

[223] F. Rougeon, P. Kourilsky, and B. Mach. Insertion of a rabbit β-globin gene sequence into an e. coli plasmid. *Nucleic acids research*, 2(12):2365, 1975.

[224] T. Maniatis, S.G. Kee, A. Efstratiadis, and F.C. Kafatos. Amplification and characterization of a [beta]-globin gene synthesized in vitro. *Cell*, 8(2):163–182, 1976.

[225] A. Efstratiadis, F.C. Kafatos, A.M. Maxam, and T. Maniatis. Enzymatic in vitro synthesis of globin genes. *Cell*, 7(2):279–288, 1976.

[226] TH Rabbitts. Bacterial cloning of plasmids carrying copies of rabbit globin messenger rna. *Nature*, 260:221–225, 1976.

[227] F. Rougeon and B. Mach. Stepwise biosynthesis in vitro of globin genes from globin mrna by dna polymerase of avian myeloblastosis virus. *Proceedings of the National Academy of Sciences of the United States of America*, 73(10):3418, 1976.

[228] P.E. Lobban and AD Kaiser. Enzymatic end-to-end joining of dna molecules* 1. *Journal of molecular biology*, 78(3):453–460, 1973.

[229] D.A. Jackson, R.H. Symons, and P. Berg. Biochemical method for inserting new genetic information into dna of simian virus 40: Circular sv40 dna molecules containing lambda phage genes and the galactose operon of escherichia coli. *Proceedings of the National Academy of Sciences*, 69(10):2904, 1972.

[230] P.C. Wensink, D.J. Finnegan, J.E. Donelson, and D.S. Hogness. A system for mapping dna sequences in the chromosomes of drosophila melanogaster. *Cell*, 3(4):315–325, 1974.

[231] A. Efstratiadis, T. Maniatis, F.C. Kafatos, A. Jeffrey, and J.N. Vournakis. Full length and discrete partial reverse transcripts of globin and chorion mrnas. *Cell*, 4(4):367–378, 1975.

[232] J. Hurwitz and J.P. Leis. Rna-dependent dna polymerase activity of rna tumor viruses i. directing influence of dna in the reaction. *Journal of Virology*, 9(1):116, 1972.

[233] J.P. Leis and J. Hurwitz. Rna-dependent dna polymerase activity of rna tumor viruses ii. directing influence of rna in the reaction. *Journal of Virology*, 9(1):130, 1972.

[234] R. Higuchi, G.V. Paddock, R. Wall, and W. Salser. A general method for cloning eukaryotic structural gene sequences. *Proceedings of the National Academy of Sciences*, 73(9):3146, 1976.

[235] M.L. Kotewicz, C.M. Sampson, J.M. D'Alessio, and G.F. Gerard. Isolation of cloned moloney murine leukemia virus reverse transcriptase lacking ribonuclease h activity. *Nucleic acids research*, 16(1):265, 1988.

[236] GF Gerard, BJ Schmidt, ML Kotewicz, and JH Campbell. cdna synthesis by moloney murine leukemia virus rnase h-minus reverse transcriptase possessing full dna polymerase activity. *Focus*, 14(91-93):91–93, 1992.

[237] M.L. Kotewicz and G.F. Gerard. Cloned genes encoding reverse transcriptase lacking rnase h activity, September 14 1993. US Patent 5,244,797.

[238] C. Coleclough and F.L. Erlitz. Use of primer-restriction-end adapters in a novel cdna cloning strategy. *Gene*, 34(2-3):305–314, 1985.

[239] U. Krawinkel and R. Zoebelein. Rapid synthesis of cdna for cloning into lambda vectors. *Nucleic acids research*, 14(4):1913, 1986.

[240] J.H. Han, C. Stratowa, and W.J. Rutter. Isolation of full-length putative rat lysophospholipase cdna using improved methods for mrna isolation and cdna cloning. *Biochemistry*, 26(6):1617–1625, 1987.

[241] H. Okayama and P. Berg. High-efficiency cloning of full-length cdna. *Molecular and Cellular Biology*, 2(2):161, 1982.

[242] H. Okayama and P. Berg. A cdna cloning vector that permits expression of cdna inserts in mammalian cells. *Molecular and Cellular Biology*, 3(2):280, 1983.

[243] H. Land, M. Grez, H. Hauser, W. Lindenmaier, and G. Sch
"utz. 5'-terminal sequences of eucaryotic mrna can be cloned with high efficiency. *Nucleic Acids Research*, 9(10):2251, 1981.

[244] P.D. Ennis, J. Zemmour, R.D. Salter, and P. Parham. Rapid cloning of hla-a, b cdna by using the polymerase chain reaction: frequency and nature of errors produced in amplification. *Proceedings of the National Academy of Sciences of the United States of America*, 87(7):2833, 1990.

[245] J. Eberwine, H. Yeh, K. Miyashiro, Y. Cao, S. Nair, R. Finnell, M. Zettel, and P. Coleman. Analysis of gene expression in single live neurons. *Proceedings of the National Academy of Sciences*, 89(7):3010, 1992.

[246] T. Tougan, D. Okuzaki, and H. Nojima. Chum-rna allows preparation of a high-quality cdna library from a single-cell quantity of mrna without pcr amplification. *Nucleic acids research*, 36(15):e92, 2008.

[247] K. Taniguchi, T. Kajiyama, and H. Kambara. Quantitative analysis of gene expression in a single cell by qPCR. *Nature Methods*, 6(7):503–506, 2009.

[248] H. Nojima and T. Tougan. Preparation of a high-quality cdna library from a single-cell quantity of mrna using chum-rna. *Methods in molecular biology (Clifton, NJ)*, 729:15, 2011.

[249] C.H. Hartmann and C.A. Klein. Gene expression profiling of single cells on large-scale oligonucleotide arrays. *Nucleic Acids Research*, 34(21):e143, 2006.

[250] Y. Cao, K.S. Wilcox, C.E. Martin, T.L. Rachinsky, J. Eberwine, and M.A. Dichter. Presence of mrna for glutamic acid decarboxylase in both excitatory and inhibitory neurons. *Proceedings of the National Academy of Sciences*, 93(18):9844, 1996.

[251] N. Chow, C. Cox, L.M. Callahan, J.M. Weimer, L.R. Guo, and P.D. Coleman. Expression profiles of multiple genes in single neurons of alzheimers disease. *Proceedings of the National Academy of Sciences of the United States of America*, 95(16):9620, 1998.

[252] P.B. Crino and J. Eberwine. Molecular characterization of the dendritic growth cone: regulated mrna transport and local protein synthesis. *Neuron*, 17(6):1173–1187, 1996.

[253] J. Phillips and J.H. Eberwine. Antisense rna amplification: a linear amplification method for analyzing the mrna population from single living cells. *Methods*, 10(3):283–288, 1996.

[254] C. Grierson and AM Hetherington. *Practical systems biology*, volume 61. Taylor & Francis Group, 2008.

[255] D.J. Surmeier, A. Reiner, M.S. Levine, and M.A. Ariano. Are neostriatal dopamine receptors co-localized? *Trends in neurosciences*, 16(8):299–305, 1993.

[256] R.N. Van Gelder, M.E. von Zastrow, A. Yool, W.C. Dement, J.D. Barchas, and J.H. Eberwine. Amplified rna synthesized from limited quantities of heterogeneous cdna. *Proceedings of the National Academy of Sciences of the United States of America*, 87(5):1663, 1990.

[257] J. Eberwine, C. Spencer, K. Miyashiro, S. Mackler, and R. Finnell. [9] complementary dna synthesis in situ: Methods and applications. *Methods in enzymology*, 216:80–100, 1992.

[258] J. Eberwine and P. Crino. Analysis of mrna populations from single live and fixed cells of the central nervous system. 1997.

[259] S.D. Ginsberg, S. Che, S.E. Counts, and E.J. Mufson. Single cell gene expression profiling in alzheimer's disease. *NeuroRx*, 3(3):302–318, 2006.

[260] A.C. Eklund, L.R. Turner, P. Chen, R.V. Jensen, et al. Replacing crna targets with cdna reduces microarray cross-hybridization. *Nature biotechnology*, 24(9):1071–1073, 2006.

[261] G. Brady and NN Iscove. Construction of cDNA libraries from single cells. *Methods in enzymology*, 225:611, 1993.

[262] G. Brady, M. Barbara, and N.N. Iscove. Representative in vitro cdna amplification from individual hemopoietic cells and colonies. *Methods Mol Cell Biol*, 2(1):17–25, 1990.

[263] L. Rambhatla, B. Patel, N. Dhanasekaran, and K.E. Latham. Analysis of G protein α subunit mRNA abundance in preimplantation mouse embryos using a rapid, quantitative RT-PCR approach. *Molecular reproduction and development*, 41(3):314–324, 1995.

[264] M. Mann, K.E. Latham, and S. Varmuza. Identification of genes showing altered expression in preimplantation and early postimplantation parthenogenetic embryos. *Developmental genetics*, 17(3):223–232, 1995.

[265] B. Zhumabayeva, L. Diatchenko, A. Chenchik, and P.D. Siebert. Use of smart-generated cdna for gene expression studies in multiple human tumors. *Biotechniques*, 30(1):158–163, 2001.

[266] T. Subkhankulova and F.J. Livesey. Comparative evaluation of linear and exponential amplification techniques for expression profiling at the single-cell level. *Genome biology*, 7(3):R18, 2006.

[267] S. Che and S.D. Ginsberg. Amplification of rna transcripts using terminal continuation. *Laboratory investigation*, 84(1):131–137, 2003.

[268] M.J. Alldred, S. Che, and S.D. Ginsberg. Terminal continuation (tc) rna amplification enables expression profiling using minute rna input obtained from mouse brain. *International journal of molecular sciences*, 9(11):2091–2104, 2008.

[269] E. Gonzalez-Roca, X. Garcia-Albéniz, S. Rodriguez-Mulero, R.R. Gomis, K. Kornacker, and H. Auer. Accurate Expression Profiling of Very Small Cell Populations. *PloS one*, 5(12):e14418, 2010.

[270] K. Miyashiro, M. Dichter, and J. Eberwine. On the nature and differential distribution of mrnas in hippocampal neurites: implications for neuronal functioning. *Proceedings of the National Academy of Sciences of the United States of America*, 91(23):10800, 1994.

[271] NA Datson, J. Van der Perk-de Jong, MP Van den Berg, ER De Kloet, and E. Vreugdenhil. Microsage: a modified procedure for serial analysis of gene expression in limited amounts of tissue. *Nucleic acids research*, 27(5):1300, 1999.

[272] B. St Croix, VE Velculescu, L. Zhang, W. Zhou, G. Traverso, B. Vogelstein, et al. Microsage detailed protocol. *Baltimore, MD: Johns Hopkins Oncology Center, Howard Hughes Medical Institute*, 2000.

[273] Y. Yabuta, K. Kurimoto, Y. Ohinata, Y. Seki, and M. Saitou. Gene expression dynamics during germline specification in mice identified by quantitative single-cell gene expression profiling. *Biology of reproduction*, 75(5):705, 2006.

[274] K. Kurimoto, Y. Yabuta, Y. Ohinata, Y. Ono, K.D. Uno, R.G. Yamada, H.R. Ueda, and M. Saitou. An improved single-cell cDNA amplification method for efficient high-density oligonucleotide microarray analysis. *Nucleic Acids Research*, 34(5):e42, 2006.

[275] V. Benes and M. Muckenthaler. Standardization of protocols in cdna microarray analysis. *Trends in Biochemical Sciences*, 28(5):244–249, 2003.

[276] F. Tang, K. Lao, and M.A. Surani. Development and applications of single-cell transcriptome analysis. *Nature Methods*, 2011.

[277] M.L. Metzker. Sequencing technologiesthe next generation. *Nature Reviews Genetics*, 11(1):31–46, 2009.

[278] F. Ozsolak, D.T. Ting, B.S. Wittner, B.W. Brannigan, S. Paul, N. Bardeesy, S. Ramaswamy, P.M. Milos, and D.A. Haber. Amplification-free digital gene expression profiling from minute cell quantities. *Nature methods*, 7(8):619–621, 2010.

[279] E. Wang, L.D. Miller, G.A. Ohnmacht, E.T. Liu, and F.M. Marincola. High-fidelity mrna amplification for gene profiling. *Electrophoresis*, 20:1224–1232, 1999.

[280] LR Baugh, AA Hill, EL Brown, and C.P. Hunter. Quantitative analysis of mrna amplification by in vitro transcription. *Nucleic Acids Research*, 29(5):e29, 2001.

[281] V. Nygaard, M. Holden, A. Løland, M. Langaas, O. Myklebost, and E. Hovig. Limitations of mRNA amplification from small-size cell samples. *Bmc Genomics*, 6(1):147, 2005.

[282] E.M. Goley, S.J. Anderson, C. Ménard, E. Chuang, X. L
"u, P.J. Tofilon, and K. Camphausen. Microarray analysis in clinical oncology: pre-clinical optimization using needle core biopsies from xenograft tumors. *BMC cancer*, 4(1):20, 2004.

[283] V. Nygaard, A. Løland, M. Holden, M. Langaas, H. Rue, F. Liu, O. Myklebost, Ø. Fodstad, E. Hovig, and B. Smith-Sørensen. Effects of mrna amplification on gene expression ratios in cdna experiments estimated by analysis of variance. *Bmc Genomics*, 4(1):11, 2003.

[284] I. Tietjen, J.M. Rihel, Y. Cao, G. Koentges, L. Zakhary, and C. Dulac. Single-cell transcriptional analysis of neuronal progenitors. *Neuron*, 38(2):161–175, 2003.

[285] F. Kamme, R. Salunga, J. Yu, D.T. Tran, J. Zhu, L. Luo, A. Bittner, H.Q. Guo, N. Miller, J. Wan, et al. Single-cell microarray analysis in hippocampus ca1: demonstration and validation of cellular heterogeneity. *The Journal of neuroscience*, 23(9):3607, 2003.

[286] C. Dulac and R. Axel. A novel family of genes encoding putative pheromone receptors in mammals. *Cell*, 83(2):195–206, 1995.

[287] J.M. Trimarchi, M.B. Stadler, B. Roska, N. Billings, B. Sun, B. Bartch, and C.L. Cepko. Molecular heterogeneity of developing retinal ganglion and amacrine cells revealed through single cell gene expression profiling. *The Journal of Comparative Neurology*, 502(6):1047–1065, 2007.

[288] F. Tang, C. Barbacioru, Y. Wang, E. Nordman, C. Lee, N. Xu, X. Wang, J. Bodeau, B.B. Tuch, A. Siddiqui, et al. mrna-seq whole-transcriptome analysis of a single cell. *Nature methods*, 6(5):377–382, 2009.

[289] M. Zirlinger. Selection and validation of microarray candidate genes from subregions and subnuclei of the brain. *Methods*, 31(4):290–300, 2003.

[290] V. Quaranta and S.P. Garbett. Not all noise is waste. *nature methods*, 7(4):269–272, 2010.

[291] A. Novick and M. Weiner. Enzyme induction as an all-or-none phenomenon. *Proceedings of the National Academy of Sciences of the United States of America*, 43(7):553, 1957.

[292] M.S.H. Ko. A stochastic model for gene induction. *Journal of theoretical biology*, 153(2):181–194, 1991.

[293] M. Zernicka-Goetz and S. Huang. Stochasticity versus determinism in development: a false dichotomy? *Nature Reviews Genetics*, 2010.

[294] E.M. Ozbudak, M. Thattai, I. Kurtser, A.D. Grossman, and A. van Oudenaarden. Regulation of noise in the expression of a single gene. *Nature genetics*, 31(1):69–73, 2002.

[295] M.B. Elowitz, A.J. Levine, E.D. Siggia, and P.S. Swain. Stochastic gene expression in a single cell. *Science*, 297(5584):1183, 2002.

[296] P.S. Swain, M.B. Elowitz, and E.D. Siggia. Intrinsic and extrinsic contributions to stochasticity in gene expression. *Proceedings of the National Academy of Sciences of the United States of America*, 99(20):12795, 2002.

[297] W.J. Blake, M. Kærn, C.R. Cantor, and J.J. Collins. Noise in eukaryotic gene expression. *Nature*, 422(6932):633–637, 2003.

[298] J.M. Raser and E.K. O'Shea. Control of stochasticity in eukaryotic gene expression. *Science*, 304(5678):1811, 2004.

[299] A. Raj and A. van Oudenaarden. Single-molecule approaches to stochastic gene expression. *Annual review of biophysics*, 38:255–270, 2009.

[300] J. Peccoud and B. Ycart. Markovian modeling of gene-product synthesis. *Theoretical population biology*, 48(2):222–234, 1995.

[301] R. Bahar, C.H. Hartmann, K.A. Rodriguez, A.D. Denny, R.A. Busuttil, M.E.T. Dollé, R.B. Calder, G.B. Chisholm, B.H. Pollock, C.A. Klein, et al. Increased cell-to-cell variation in gene expression in ageing mouse heart. *Nature*, 441(7096):1011–1014, 2006.

[302] K.W. Yau and R.C. Hardie. Phototransduction motifs and variations. *Cell*, 139(2):246–264, 2009.

[303] H.H. Chang, M. Hemberg, M. Barahona, D.E. Ingber, and S. Huang. Transcriptome-wide noise controls lineage choice in mammalian progenitor cells. *Nature*, 453(7194):544–547, 2008.

[304] D.P. Costello. Experimental studies of germinal localization in Nereis. I. The development of isolated blastomeres. *Journal of Experimental Zoology*, 100(1):19–66, 1945.

[305] A.H.L. Fischer, T. Henrich, and D. Arendt. The normal development of platynereis dumerilii (nereididae, annelida). *Frontiers in Zoology*, 7(1):31, 2010.

[306] E.B. Wilson. *The cell-lineage of Nereis*. 1892.

[307] W.S. Engstrom. Removal of the fertilization membrane of fertilized eggs of urechis caupo and development of" membraneless" embryos. *Biological Bulletin*, 140(3):369–375, 1971.

[308] Je. Mechanism of phototaxis in marine zooplankton.

[309] AK Dondua, AWC Dorresteijn, RP Kostyuchenko, Z.E. Fedorova, and A. Fisher. Effect of aphidicolin on the differentiation of trochoblasts in early ontogenesis of polychaetes. *Russian Journal of Development Biology*, 27(6):341–347, 1996.

[310] RP Kostyuchenko and AK Dondua. Development of the prototroch in embryogenesis of nereis virens (polychaeta). *Russian Journal of Developmental Biology*, 37(2):69–76, 2006.

[311] L.N. Curtis and J.L. Carson. Computer-assisted video measurement of inhibition of ciliary beat frequency of human nasal epithelium in vitro by xylometazoline. *Journal of pharmacological and toxicological methods*, 28(1):1–7, 1992.

[312] M Schroer. Proliferation und Charakterisierung von Elaeocyten bei Platynereis dumerilii (Annelida, Polychaeta). Master's thesis, 2004.

[313] K. Tessmar-Raible, F. Raible, F. Christodoulou, K. Guy, M. Rembold, H. Hausen, and D. Arendt. Conserved sensory-neurosecretory cell types in annelid and fish forebrain: insights into hypothalamus evolution. *Cell*, 129(7):1389–1400, 2007.

[314] B. Altincicek and A. Vilcinskas. Analysis of the immune-related transcriptome of a lophotrochozoan model, the marine annelid platynereis dumerilii. *Frontiers in zoology*, 4(1):18, 2007.

[315] M. Bengtsson, M. Hemberg, P. Rorsman, and A. Ståhlberg. Quantification of mrna in single cells and modelling of rt-qpcr induced noise. *BMC molecular biology*, 9(1):63, 2008.

[316] F. Tang, C. Barbacioru, E. Nordman, B. Li, N. Xu, V.I. Bashkirov, K. Lao, and M.A. Surani. Rna-seq analysis to capture the transcriptome landscape of a single cell. *Nature Protocols*, 5(3):516–535, 2010.

[317] J. Jolly. *Sur la durée de la vie et de la multiplication des cellules animales en dehors de l'organisme*. 1903.

[318] J.P. Mather and P.E. Roberts. *Introduction to cell and tissue culture: theory and technique*. Plenum Pub Corp, 1998.

[319] R.G. Harrison. The outgrowth of the nerve fiber as a mode of protoplasmic movement. *Journal of Experimental Zoology*, 9(4):787–846, 1910.

[320] A. Carrel. On the permanent life of tissues outside of the organism. *The Journal of Experimental Medicine*, 15(5):516, 1912.

[321] A. Carrel and C.A. Lindbergh. The culture of organs. *The American Journal of the Medical Sciences*, 196(5):732, 1938.

[322] A. Carrel and C.A. Lindbergh. The culture of whole organs. *Science*, 81(2112):621, 1935.

[323] A. Carrel and A.H. Ebeling. Pure cultures of large mononuclear leucocytes. *The Journal of experimental medicine*, 36(4):365, 1922.

[324] C.A. Lindbergh. An apparatus for the culture of whole organs. *The Journal of Experimental Medicine*, 62(3):409, 1935.

[325] G.O. Gey, G.E. Seegar, and L.M. Hellman. The production of a gonadotrophic substance (prolan) by placental cells in tissue culture. *Science*, 88(2283):306, 1938.

[326] AE Feller, J.F. Enders, and TH Weller. The prolonged coexistence of vaccinia virus in high titre and living cells in roller tube cultures of chick embryonic tissues. *The Journal of Experimental Medicine*, 72(4):367, 1940.

[327] S. Fazekas de St Groth and DO White. An improved assay for the infectivity of influenza viruses. *Journal of Hygiene*, 56(01):151–162, 1958.

[328] P.R. White. Prolonged survival of excised animal tissues in vitro in nutrients of known constitution. *Journal of Cellular and Comparative Physiology*, 34(2):221–241, 1949.

[329] H. Eagle and K. Habel. The nutritional requirements for the propagation of poliomyelitis virus by the hela cell. *The Journal of Experimental Medicine*, 104(2):271, 1956.

[330] H. Eagle, V.I. Oyama, M. Levy, C.L. Horton, and R. Fleischman. The growth response of mammalian cells in tissue culture to l-glutamine and l-glutamic acid. *Journal of Biological Chemistry*, 218(2):607, 1956.

[331] H. Eagle. The minimum vitamin requirements of the l and hela cells in tissue culture, the production of specific vitamin deficiencies, and their cure. *The Journal of Experimental Medicine*, 102(5):595, 1955.

[332] H. Eagle. The specific amino acid requirements of a human carcinoma cell (strain hela) in tissue culture. *The Journal of Experimental Medicine*, 102(1):37, 1955.

[333] H. Eagle. Nutrition needs of mammalian cells in tissue culture. *Science*, 122(3168):501, 1955.

[334] J.F. Morgan, H.J. Morton, and R.C. Parker. Nutrition of animal cells in tissue culture. l initial studies on a synthetic medium. *Proceedings of the Society for Experimental Biology and Medicine*, 73:1–8, 1950.

[335] R.G. Ham. Clonal growth of mammalian cells in a chemically defined, synthetic medium. *Proceedings of the National Academy of Sciences of the United States of America*, 53(2):288, 1965.

[336] S. Wyatt. Culture in vitro of tissue from the silkworm, bombyx mori l. *The Journal of General Physiology*, 39(6):841, 1956.

[337] D.E. Lynn. Development of insect cell lines: Virus susceptibility and applicability to prawn cell culture. *Methods in cell science*, 21(4):173–181, 1999.

[338] J. Mitsuhashi and K. Maramorosch. Leafhopper tissue culture. *Embryonic nymphal and imaginal tissues from aseptic insects. Contribution Boyce Thompson Inst*, 22:435–460, 1964.

[339] J. Mitsuhashi, K. Maramorosch, and F. Mahmood. Sterile techniques for rearing and cell culture of leafhopper vectors of plant pathogens. *Maintenance of human, animal, and plant pathogen vectors.*, pages 307–319, 1999.

[340] J. Mitsuhashi and K. Maramorosch. Aseptic cultivation of four virus transmitting species of leafhoppers (cicadellidae). *Contrib. Boyce Thompson Inst*, 22:165–173, 1963.

[341] A. Rosenfield. Marine invertebrate cell culture: breaking the barriers. *NOAA Technical Memorandum NMFS-F/NEC-98*, 25, 1993.

[342] B. Rinkevich. Cell cultures from marine invertebrates: obstacles, new approaches and recent improvements. *Progress in Industrial Microbiology*, 35:133–153, 1999.

[343] B. Rinkevich. Marine invertebrate cell cultures: new millennium trends. *Marine Biotechnology*, 7(5):429–439, 2005.

[344] S. O'Boyle, G. McDermott, and R. Wilkes. Dissolved oxygen levels in estuarine and coastal waters around ireland. *Marine pollution bulletin*, 58(11):1657–1663, 2009.

[345] NOG Jørgensen. Uptake of l-valine and other amino acids by the polychaete nereis virens. *Marine Biology*, 52(1):45–52, 1979.

[346] SD Batten and RN Bamber. The effects of acidified seawater on the polychaete nereis virens sars, 1835. *Marine pollution bulletin*, 32(3):283–287, 1996.

[347] I. Kuhlmann. The prophylactic use of antibiotics in cell culture. *Cytotechnology*, 19(2):95–105, 1995.

[348] B.F. Chow and C.M. McKee. Interaction between crystalline penicillin and human plasma proteins. *Science*, 101(2612):67, 1945.

[349] GN Rolinson and R. Sutherland. The binding of antibiotics to serum proteins. *British Journal of Pharmacology and Chemotherapy*, 25(3):638, 1965.

[350] D.A. Laska, P.D. Williams, S.L. White, C.A. Thompson, and D.M. Hoover. In vitro correlation of ultrastructural morphology and creatine phosphokinase release in l6 skeletal muscle cells after exposure to parenteral antibiotics. *In Vitro Cellular & Developmental Biology-Plant*, 26(4):393–398, 1990.

[351] A. Lange and G. Assmann. Penetration of penicillin g and ampicillin into mice fibroblasts (earle's strain l). effects of temperature and serum content of culture media]. *Zeitschrift f*
"ur medizinische Mikrobiologie und Immunologie, 153(1):60, 1966.

[352] K.A. Neftel and U. Hubscher. Effects of beta-lactam antibiotics on proliferating eucaryotic cells. *Antimicrobial agents and chemotherapy*, 31(11):1657, 1987.

[353] P. Katharios, R.P. Smullen, and V. Inglis. The use of the polychaete worm nereis virens eggs as vehicle for the delivery of oxytetracycline in solea solea larvae. *Aquaculture*, 243(1-4):1–7, 2005.

[354] G. Repetto, A. del Peso, and J.L. Zurita. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nature Protocols*, 3(7):1125–1131, 2008.

[355] E. Mialhe, V. Boulo, and H. Grizel. Bivale mollusc cell culture. *American Fisheries Society Special Publication*, 18:311–315, 1988.

[356] Q. Zhao, W. Zhang, M. Jin, X. Yu, and M. Deng. Formulation of a basal medium for primary cell culture of the marine sponge hymeniacidon perleve. *Biotechnology progress*, 21(3):1008–1012, 2005.

[357] C. Vago and S. Chastang. Culture of oyster tissues. *Comptes rendus hebdomadaires des sÃŠances de l'AcadÃŠmie des sciences*, 250:2751, 1960.

[358] M. Klautau, M.R. Custodio, and R. Borojevic. Cell cultures of sponges clathrina and polymastia. *In Vitro Cellular & Developmental Biology-Animal*, 29(2):97–99, 1993.

[359] A. Machii and KT Wada. Some marine invertebrates tissue culture. *Invertebrate cell system applications*, 2:226–233, 1989.

[360] P. OLIVE, S. CRAIG, and G. RUTHERFORD. Enhanced aquaculture feeds, February 16 2006. WO Patent WO/2006/016,135.

[361] NA Odintsova and AV Khomenko. Primary cell culture from embryos of the japanese scallop mizuchopecten yessoensis (bivalvia). *Cytotechnology*, 6(1):49–54, 1991.

[362] P. L
"ahdesm
"aki. Biosynthesis of taurine peptides in brain cytoplasmic fraction in vitro. *International journal of neuroscience*, 37(1):79–84, 1987.

[363] C.J. Costa, S.K. Pierce, and M. Warren. The intracellular mechanism of salinity tolerance in polychaetes: volume regulation by isolated glycera dibranchiata red coelomocytes. *The Biological Bulletin*, 159(3):626, 1980.

[364] T. Harder and P.Y. Qian. Induction of larval attachment and metamorphosis in the serpulid polychaete hydroides elegans by dissolved free amino acids: isolation and identification. *Marine Ecology Progress Series*, 179:259–271, 1999.

[365] Sigma aldrich- on-line media guide.

[366] J.E. Bottenstein. *Cell culture in the neurosciences*. 1985.

[367] N. Rebscher, F. Zelada-González, TU Banisch, F. Raible, and D. Arendt. Vasa unveils a common origin of germ cells and of somatic stem cells from the posterior growth zone in the polychaete platynereis dumerilii. *Developmental biology*, 306(2):599–611, 2007.

[368] B. Prud'homme, R. de Rosa, D. Arendt, J.F. Julien, R. Pajaziti, A.W.C. Dorresteijn, A. Adoutte, J. Wittbrodt, and G. Balavoine. Arthropod-like expression patterns of engrailed and wingless in the annelid platynereis dumerilii suggest a role in segment formation. *Current biology*, 13(21):1876–1881, 2003.

[369] L. Christiaen, E. Wagner, W. Shi, and M. Levine. Electroporation of transgenic dnas in the sea squirt ciona. *Cold Spring Harbor protocols*, 2009(12):pdb–prot5345, 2009.

[370] B. Neumann, M. Held, U. Liebel, H. Erfle, P. Rogers, R. Pepperkok, and J. Ellenberg. High-throughput rnai screening by time-lapse imaging of live human cells. *Nature methods*, 3(5):385–390, 2006.

[371] S.M. Albelda and C.A. Buck. Integrins and other cell adhesion molecules. *The FASEB journal*, 4(11):2868, 1990.

[372] S. Miyamoto, B.E.N.Z. KATHZ, R.M. Lafrenie, and K.M. Yamada. Fibronectin and integrins in cell adhesion, signaling, and morphogenesis. *Annals of the New York Academy of Sciences*, 857(1):119–129, 1998.

[373] M. Durchon and F. Schaller. Recherches endocrinologiques en culture organotypique chez les annélides polychčtes. *General and Comparative Endocrinology*, 4(4):427–432, 1964.

[374] NOG Jorgensen and E. Kristensen. Uptake of amino acids by three species of nereis (annelida: Polychaeta). i. transport kinetics and net uptake from natural concentrations. *Mar. Ecol. Prog. Ser*, 3:329–340, 1980.