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**3D characterization of vascular architectures
in experimental tumors based on LSM microscopy
and simulation of the oxygen distributions**

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Zusammenfassung

Tumore weisen chaotische vaskuläre Netzwerke auf, die hypoxische und damit strahlenresistentere Subareale verursachen, welche den Erfolg einer Strahlentherapie einschränken. In dieser Arbeit wurde eine Analyse-Pipeline für die 3D-Lichtscheiben-Fluoreszenzmikroskopie (LSFM) etabliert, um vollständige Tumor-Gefäßnetzwerke zu charakterisieren. Zwei Tumorlinien mit unterschiedlicher Strahlenempfindlichkeit wurden mit Protonen in zwei Fraktionierungsschemata bestrahlt und mit unbehandelten Kontrolltumoren und Gehirnprouben verglichen.

Für die Charakterisierung des vaskulären Netzwerks wurden drei Parameterklassen verwendet: (i) Parameter, die mit 2D-Histologie und 3D-LSFM messbar waren, zeigten eine gute Übereinstimmung zwischen den beiden Methoden. (ii) Konventionelle 3D-Parameter, z. B. die Anzahl der miteinander verbundenen Gefäßverzweigungen, waren nützlich, um verschiedene Gewebetypen zu differenzieren. (iii) Graphentheoretische Netzwerkparameter, z. B. die Graphengröße, konnten zur Gewebeunterscheidung verwendet werden und zeigten eine strahleninduzierte Abnahme der globalen Netzwerkeffizienz.

Darüber hinaus wurde ein bereits existierender Algorithmus zur Simulation des Sauerstofftransports numerisch optimiert und einem Benchmarking unterzogen. Die simulierten Sauerstoffverteilungen innerhalb der experimentellen Gewebeprouben ergaben eine erhöhte Oxygenierung nach der Bestrahlung, was auf eine Reoxygenierung hinweist. Diese Ergebnisse, die auch die gewebespezifischen Eigenschaften der charakterisierten Gefäßarchitekturen berücksichtigen, können zur Verbesserung von Simulationen für die Strahlenreaktion von hypoxischen Tumoren verwendet werden.

Summary

Tumors exhibit chaotic vascular networks, causing hypoxic and thus more radioresistant subregions, which limit the success of radiotherapy. In this work, an analysis pipeline for 3D light-sheet fluorescence microscope (LSFM) images was established to characterize entire tumoral vascular networks. Two tumor lines with different radiosensitivities were irradiated with protons in two fractionation schemes and compared to untreated control tumors and brain samples.

The vascular network characterization employed three parameter classes: (i) Parameters that were measurable on 2D histology and 3D LSFM showed a good agreement between the two methods. (ii) Conventional 3D quantities, e.g., the number of interconnected vascular branches, were useful to separate different tissue types. (iii) Graph-theoretic network parameters, e.g., the graph size, could be used for tissue discrimination and revealed a radiation-induced decrease in the global network efficiency.

Furthermore, an existing oxygen transport simulation algorithm was numerically optimized and benchmarked. The simulated oxygen distributions within the experimental tissue samples revealed increased oxygenation after irradiation, indicating re-oxygenation. These results, which also account for the tissue-specific properties of the characterized vascular architectures, may be used to improve simulations for the radiation response of hypoxic tumors.

*“Not only does God play dice, but ...
he sometimes throws them where they cannot be seen.”*

Stephen Hawking

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List of Abbreviations

Adenosine triphosphate (ATP)	30
Boundary condition (BC)	22
Confidence interval (CI).....	33
Courant-Friedrichs-Lewy (CFL) condition	60
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1. Introduction

Cancer has always been present in the history of humankind. The first documented cases of cancer were recorded in the *Ebers Papyrus*, an Egyptian medical manuscript from the 16th century BC. The Ebers Papyrus describes eight cases of mammary tumors that could only be treated palliatively at that time [1], [2]. The name *cancer* was later given to this disease by Hippocrates in the fourth century BC. For the naming, he either adopted the name from the vernacular [2] or drew parallels between the shape of the animal cancer and the superficial growths of mammary carcinomas [3].

For a long time, though, the underlying causes of cancer remained unclear and it was assumed that cancer was induced by external pathogens. It was not until the 19th century that the cellular pathologist Rudolf Virchow discovered, with the help of the newly developed *Abbe microscope*, that cancer cells develop from healthy cells [2], [3]. This discovery initiated the development of new therapies in the first half of the 20th century that complemented surgical removal, the only treatment available until then. The two new types of treatment were radiotherapy and chemotherapy. The former was based on the discovery of X-rays by Wilhelm Conrad Röntgen in 1895 [4], and the latter was pioneered in 1909 by Paul Ehrlich, who developed the first therapeutic agent specifically acting against syphilis [3].

In the second half of the 20th century, the search for ionizing particles suitable for radiotherapy began, starting with mouse experiments at the *Crocker Laboratory* (Berkeley, California). The Berkeley experiments in 1940 showed favorable biological effects on tumors irradiated with neutrons compared to the X-rays [5] that had been used exclusively until then. Besides that, protons and heavy ions were also found to be beneficial compared to photons due to their highly localized dose deposition and higher biological effectiveness [6].

Meanwhile, research began to investigate methods to use the body's immune system to fight cancer. Harald zur Hausen's research group, for example, has been studying *human papillomaviruses* (HPV) as a trigger for cervical cancer since the 1970s. Their work led to the release of the first vaccine against HPV in 2006 to prevent the development of cancer [3]. Other areas of research in this field also include immunotherapies.

In radiotherapy, significant methodological advancements have been made over the past 126 years, beginning with the discovery of X-rays, and culminating in today's personalized high-precision, intensity-modulated, image-guided 3D irradiation. However, there are still unresolved questions about the origin and effective treatment of cancer that require further research. Both inter- and intratumoral variations in the tumor microenvironment play an important role in explaining the heterogeneous response to radiotherapy, which still limits the cure rate.

A lack of molecular oxygen in cells (*hypoxia*), for example, occurs in most tumors above a certain size and is caused by the high proliferative activity of tumors and their inadequate vascular architecture. Hypoxia leads to malignant progression and is a negative predictive factor for local control [7]–[9], disease-free [10]–[12], and overall survival [11], [13]–[15]. Hypoxia is “a characteristic feature of locally advanced solid tumors” [16] caused by an imbalance of oxygen supply and demand. Reasons for this imbalance include severely abnormal microvasculature, unstable blood flow, and high interstitial pressure due to rapid proliferation and angiogenesis. Hypoxia leads to an increase in chemoresistance, radioresistance, invasiveness, metastasis, resistance to cell death, altered metabolism, and genomic instability [17]. This usually affects only a minority of tumor cells, but these are critical for prognosis [18] and therefore must be specifically addressed in therapy. On the other hand, hypoxia also has some anti-proliferation effects, such as limiting proliferation and differentiation of the cells, which may even lead to apoptosis and necrosis [18]. This affects most tumor cells and is therefore beneficial for treatment.

Hypoxia also presents a problem at different spatial scales, as illustrated in Figure 1.1: The biochemical hypoxic response occurs at a micrometer scale. At this resolution, the response can only be measured invasively in real-time with oxygen electrodes *in vivo* or with a time delay of hours after biopsy with *immuno-histochemical* (ICH) markers. A molecular radiosensitizer and biomarker useful for detecting hypoxia, for example, is *pimonidazole*, which has been shown to be a “significant determinant of local control after fractionated irradiation” in a preclinical model for human *head and neck squamous cell carcinoma* (HNSCC) [19]. However, clinically used non-invasive imaging modalities such as *magnetic resonance imaging* (MRI) or *positron emission tomography* (PET) are only able to detect hypoxia at the millimeter scale. A similar technical accuracy applies to treatment planning, but methods for clinical targeting of hypoxia such as dose painting are limited even to the centimeter range [20].

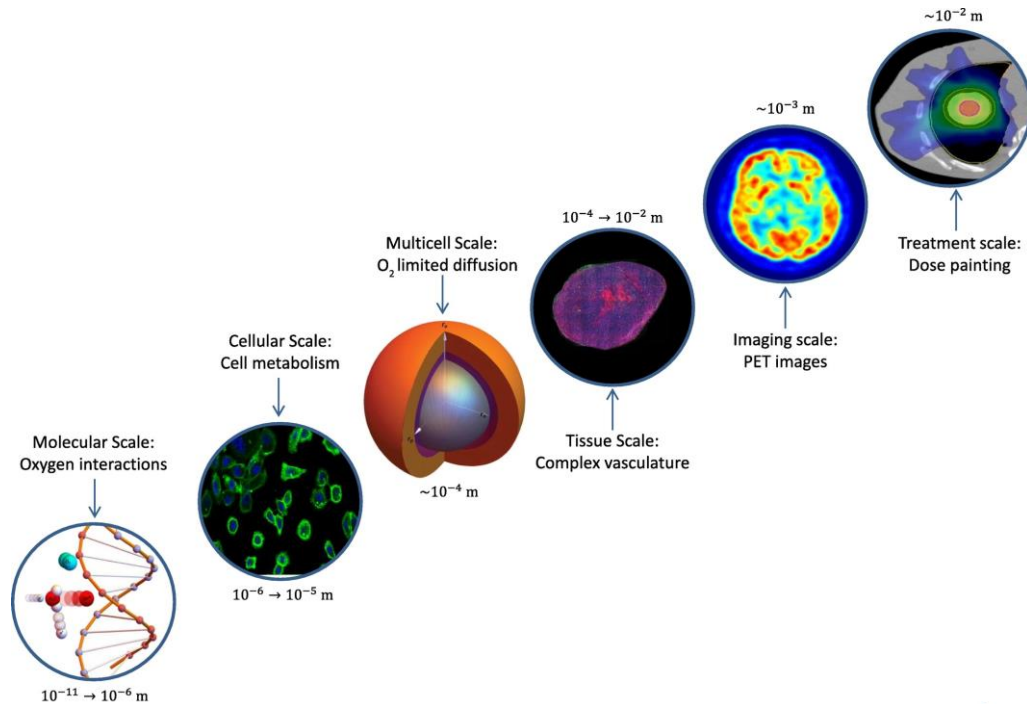


Figure 1.1. Hypoxia in tumors limits treatment success at different spatial scales. Illustration modified from [20], licensed under CC BY 4.0¹.

Besides that, translation of measured hypoxic signatures into clinically meaningful information is complicated by differences in the expression of hypoxia among different methods. The methods are distinguished by their spatial and temporal information and also in their prognostic value: Eppendorf oxygen electrode measurements showed *in vivo* that hypoxia on the microscopic scale is a prognostic factor [21]. Similarly, the hypoxic volume of ICH-stained sections was shown to be predictive, but the hypoxic fraction itself had less significance than the coupled value with the whole tumor volume [22]. However, Horsman et al. [23] found no direct correlations between oxygenations measured with oxygen electrodes and IHC, but only trends between lower oxygen tensions and higher hypoxic fractions. Therefore, these two methods characterizing hypoxia at the microscopic scale are not directly comparable. The same applies to comparisons of Eppendorf electrode measurements with other *in vivo* methods, e.g., 18F-FMISO-PET. Electrode measurements usually show more hypoxic tumors than PET values in this comparison [24]. Moreover, a direct correlation of both methods was not successful [24], [25], or only using a robotic 3D tracking of the OxyLite oxygen electrode [26].

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One way to address this multi-scale and multi-modality problem is to use biomathematical models. They can help to bridge the resolution gap between clinical and pre-clinical imaging and to predict clinical outcomes for different tumor models, radiation regimens, etc. This enables the identification and separate investigation of the underlying biological effects.

In the present work, the in-house developed *tumor oxygenation model* (TOM) [27] is used to solve the *diffusion-reaction equation* (DRE) for oxygen transport within tumor tissue. The TOM was initially designed to calculate the spatial oxygen distribution based on simplified synthetic vascular architectures with parallel linear capillaries. The calculations are performed on a microscopic scale and can therefore be used to predict radiation-induced cell killing. By downscaling the resulting spatial oxygen distributions, inferences can be made about the oxygenation at a coarser resolution. Besides that, the response of the whole tumor to different radiotherapy schedules can be simulated with the in-house developed *tumor response model* (TRM) [28] using the oxygen distributions generated with the TOM as input.

Based on this previous work by Espinoza et al. on the TOM [27], this thesis focuses on validating the model using numerical simulations as well as novel preclinical experimental techniques. Specifically, this work contributes to the following three new insights:

- (i) The mathematical validation of the TOM performed in this work aimed to increase the accuracy of the simulated oxygen distributions. As a result, a more accurate calculation of the oxygen histograms was made possible, which also improved the numerical precision of the tumor response simulations with the TRM.
- (ii) A new semi-automated workflow was established to analyze experimental vascular architectures of whole extracted tumor xenografts measured by 3D *light-sheet fluorescence microscopy* (LSFM). This workflow allowed the study of different tumor lines in terms of inter- and intra-tumoral heterogeneity of radiosensitivity and the analysis of changes in vascular architecture after the application of different irradiation schemes. In addition, differences in vascular architecture between various tumor tissues and normal brain tissue were characterized.
- (iii) Based on the experimentally reconstructed 3D vascular architecture for the different tissue samples, the oxygen distributions were simulated with the TOM. Thus, the vascular network was used as a surrogate parameter to assess tumor hypoxia and the resulting change in radiosensitivity.

2. Materials and Methods

In this chapter, the biological bases of tumor development and the response to radiation therapy are presented. Based on this, the subsequent section explains the radiobiological modeling of tumors, which is used in the in-house developed *tumor oxygenation model* (TOM). The last section then describes the key question of this thesis: Is the reduced complexity of the vascular architecture used in the TOM able to accurately reflect the main features of vascular architectures and the resulting oxygen distributions of real tissues? To this end, the experimental methods of 3D tissue preparation and analysis of the vascular architecture are described, which were performed for mouse xenografts of human *head and neck squamous cell carcinoma* (HNSCC), rat adenocarcinoma, and normal rat brain tissue.

2.1. Biological basics of radiation therapy

The interactions of ionizing radiation with matter, the importance of oxygen for tumor development, and the radiation response of tumors are discussed in the following.

2.1.1. Interaction of ionizing radiation with matter

Ionizing radiation has an energy of more than five electron volts and is, therefore, able to ionize atoms or molecules, so that they release an electron. Typical energies in radiodiagnostics are between 10 keV and up to 1 MeV, while energies above 1 MeV are used in radiotherapy. Besides ionization, this type of radiation can also change molecules in other ways, e.g., by breaking chemical bonds, which may lead to a loss of cell function.

Direct and indirect ionizing radiation

A distinction is made between directly and indirectly ionizing radiation. *Directly ionizing radiation* consists of charged particles, e.g., electrons, protons or heavier charged particles, which directly cause ionizations of atoms or molecules. *Indirectly ionizing radiation*, on the other hand, describes the interaction of uncharged particles, such as photons or neutrons, which, in contrast, react in an additional primary interaction. In this primary interaction, charged particles (electrons) are released, which in turn interact as directly ionizing radiation. In both cases, the charged particles generate

highly reactive free radicals, which subsequently damage biomolecules, especially the *deoxyribonucleic acid* (DNA).

Interaction of charged particles with matter

In the present work, protons are used for the mouse irradiations, so the focus in the following will be on charged particles.

Interaction processes

As charged particles pass through matter, basically five different types of interaction processes can be observed, which can be grouped into four classes with the size of the interaction parameter b serving as a divider [29]:

- *Soft collisions* occur for large interaction parameters b that are considerably larger than the atom radius r_{Atom} ($b \gg r_{\text{atom}}$). Thereby, a small energy amount is transferred to the absorber atom that interacts as a recoil atom in a single collision. This results in excitations or ionizations of the outer atomic hull.
- *Hard collisions* happen for $b \approx a$, whereas a secondary (δ -) electron with a significant amount of energy is ejected after a binary collision, which can then also cause multiple ionizations in the absorber.
- For small interactions parameters ($b \ll r_{\text{atom}}$), either *elastic scattering at the nucleus* or *radiative stopping* at the nucleus take place. The elastic scattering is also referred to as *nuclear stopping* since the nucleus acts as a recoil nucleus. In contrast, the radiative stopping results in the generation of *bremsstrahlung* (γ -rays).
- Interaction parameters with a size in the order of the atomic radius ($b \approx r_{\text{atom}}$) lead to nuclear interactions, resulting in fragmentations of the target nucleus.

Most of the energy is transferred in the bremsstrahlung reaction and the hull electron reactions.

Stopping power

The *total stopping power* S is used to describe the energy loss of the particles during their passage through matter. The energy loss and occurs in form of multiple interactions with the atoms or molecules of the material and is dependent on the energy and type of the incident particle and the type and density of the absorber material [30]. The total stopping power depends on the collision stopping power, and on the radiative stopping power and can be expressed as.

$$S = \frac{dE}{dx} = \left(\frac{dE}{dx} \right)_{\text{col}} + \left(\frac{dE}{dx} \right)_{\text{rad}}. \quad (2.1)$$

Equation (2.1) thus describes the total energy loss dE of a charged particle along its path dx through a material, as sum of the two processes:

- The *collision stopping power*, also known as *electronic stopping power* for heavy charged particles (HCP), refers to ionizations through interactions of charged particles with orbital electrons [29].
- In contrast, the *radiative stopping power* describes interactions of the particles with atomic nuclei [29].

The energy loss for therapeutic ion beams is dominated by the collision stopping power, which can be expressed with the *Bethe-Bloch formula* as

$$S_{col} = -\left(\frac{dE}{dx}\right)_{col} = \rho \cdot 4\pi r_e^2 \cdot m_e c^2 \cdot \frac{Z}{u \cdot A} \cdot z^2 \cdot \frac{1}{\beta^2} \cdot R_{col}(\beta). \quad (2.2)$$

Thereby ρ denotes the absorber's density, r_e the electron radius, $m_e c^2$ the electron rest energy, Z the absorber's atomic number, u the atomic mass unit, A the absorber's mass number, z the particle's charge, β the relativistic velocity relation and R_{col} a residual function for additional dependencies.

The energy loss can be expressed as a depth-dose curve, which shows the deposited dose in dependence on the depth (e.g., in water as in Figure 2.1). The graphic shows that charged particles such as protons or heavier nuclei have an advantageous physical dose distribution in the tissue with a so-called "Bragg peak" at the dose maximum compared to photons, which show an exponential decay after a build-up region. This enables dose escalation in the tumor with charged particles while sparing normal tissue.

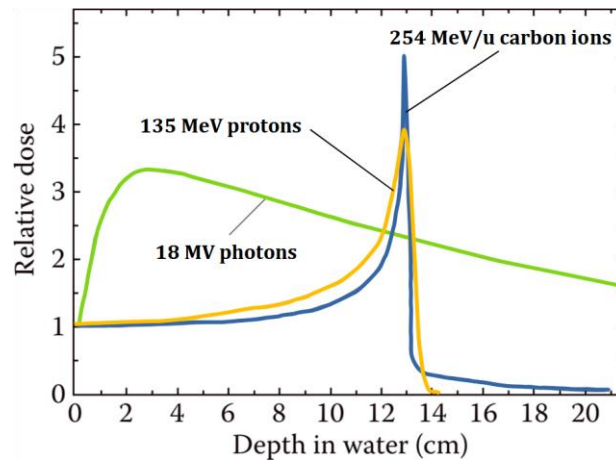


Figure 2.1. Depth-dose profiles of photons, protons, and carbon ions. Adapted from [31] with permission.

Linear energy transfer

The *linear energy transfer* (LET)

$$L = \frac{dE}{dx} \quad (2.3)$$

describes the energy dE that is transferred to the material by secondary electrons at the depth between x and $x + dx$. Depending on the LET, the radiation is categorized as low LET, i.e., sparsely ionizing (e.g., X- or γ -rays) and high LET radiation, i.e., densely ionizing (e.g., neutrons, alpha particles).

The LET may be truncated below a certain energy threshold Δ and then becomes the *restricted electronic stopping power*:

$$L_{\Delta} = \frac{dE_{\Delta}}{dx}. \quad (2.4)$$

Absorbed dose

The interaction of radiation with matter leads to the absorption of energy in the material. The *total energy* ϵ is deposited by many individual interactions, resulting in a stochastic energy deposition pattern. This stochastic process can be described by a probability distribution that reaches its expected value, the *mean total energy* $\bar{\epsilon}$ for many interaction events. The quantity $\bar{\epsilon}$ is thus suitable to define the macroscopic quantity *absorbed energy dose* (in short form *dose*) as

$$D = \frac{d\bar{\epsilon}}{dm}. \quad (2.5)$$

The unit of dose is Gray (1 Gy = 1 J/kg), named after L.H. Gray, who studied the tissue-damaging properties of ionizing radiation. Although absorbed dose is a purely physical quantity, it is generally assumed in radiotherapy and radiobiology that the effect of radiation on biological systems correlates with absorbed dose. However, since this relationship is non-linear and depends on many other physical and biological parameters, dedicated radiobiological response models have been developed.

2.1.2. Oxygenation of tumors

Tumor characteristics

Tumors in the narrower sense are two types of tissue alterations that result from uncontrolled cell growth. As a result of these neoplasms, i.e. tissue growths, tumors exhibit characteristic biological features, such as resistance to cell death, induced angiogenesis, i.e. new vessel formation, and activation of invasion and metastasis [32].

Thereby, tumors are distinguished between benign and malignant growths, with malignant tumors being the second most common cause of death in industrialized countries. Tumor progression is influenced on the one hand by tumor cytology and on the other hand by the tumor micro-environment (TME) [33], which includes, for example, the microvascular network and thus the supply of oxygen and nutrients.

Oxygen transport to tissue

Transport in the vascular network

Oxygen is taken up by the lungs from the air that has an oxygen volume fraction of 21 %, i.e., a partial tension of 160 mmHg. The initial oxygen tension in the pulmonary alveoli of 105 mmHg decreases during the transport to the organs via the hierarchical vascular network. Thus, the tension drops to 100 mmHg in the arterial blood and subsequently to 40 mmHg in the capillaries [34], [35].

Thereby, capillaries are microvessels responsible for most of the oxygen transport to tissues. A typical capillary has a diameter of 5 – 8 μm [36] with a wider range for carcinomas, e.g. a mean diameter of 20 μm for colorectal adenocarcinomas [37]. Tumor capillaries have a highly variable oxygen tension that is typically assumed to average around 40 mmHg [38].

Oxygen supply to the tissue

The oxygen supply to the tumor depends on two mechanisms, the *vascular architecture*, and its *perfusion*. The *vascular architecture* depends on parameters such as vessel topology, density, and diameter, which characterize the hierarchical structure of the network. Tumors, as discussed in the next section, form a chaotic vascular network due to rapid proliferation and angiogenesis, which limits the functionality of this metabolite transport system.

Perfusion, on the other hand, describes the amount of blood (and thus oxygen) transported through the capillaries per unit of time, including oxygen tension and blood oxygen content. Oxygen transport is related to the hemoglobin concentration in the blood since most oxygen is bound to hemoglobin (98.5 %) and only a small portion of 1.5 % is physically dissolved [39, p. 657]. Compared to the peritumoral normal tissue, tumor perfusion rates can be significantly reduced, mainly due to vessel hyperpermeability (“leakiness”), tortuosity (degree of twist), and compression due to the physical stress of the solid tumor components [40].

Vascular architecture

In general, the vascular network of tumors develops rapidly and exhibits a chaotic and primitive structure [41]. This vascular growth process is called *angiogenesis* and is one of the hallmarks of cancer [32]. Angiogenesis occurs on the time scale of days [42] and is induced by tumors to meet the high demand for nutrients and oxygen to support their high turnover and proliferation rates. In this work, the following vascular parameters are used to quantify the vascular architecture of tumors

The *vascular fraction* (VF)

$$VF = \frac{V_{all\ cap}}{V_{total}} \quad (2.6)$$

is defined as the ratio of the volume of all capillaries $V_{all\ cap}$ and the total volume V_{total} and ranges typically between 1 and 10 %. The number of capillaries N in the volume can be calculated from $V_{all\ cap}$ using the average volume V_{cap} of each capillary:

$$N = \frac{V_{all\ cap}}{V_{cap}}. \quad (2.7)$$

Equation (2.6) can then be rewritten as

$$VF = N \frac{V_{cap}}{V_{total}} \quad (2.8)$$

using the number of capillaries N with the average volume V_{cap} of each capillary.

For a given *capillary radius* r , the VF is reflected by the total length $l_{all\ cap}$ of the capillary network. The total length may be used to characterize the network in terms of the *vascular length density*:

$$VLD = \frac{l_{all\ cap}}{V_{total}}. \quad (2.9)$$

The *vascular density* (VD) may be defined as

$$VD = \frac{N_{all\ cap}}{V_{total}} \quad (2.10)$$

and scales with the VF but is independent of the capillary radius.

The *inter-vessel distance* (IVD) is defined as the shortest distance of a capillary to the nearest adjacent capillary and decreases with increasing VF. The IVD distribution, unlike the VF, emphasizes the capillary distribution, i.e., the local capillary density, which also depends on the number of vessel branches. The IVD of normal tissues is usually smaller than the maximum diffusion distance, resulting in a good oxygen supply to all cells. Tumors, in contrast, have large IVDs in many cases and therefore often show areas of poor oxygenation and even hypoxia [43].

Hypoxia

Hypoxia is caused by an imbalance between oxygen supply to the tissue and oxygen consumption [44], mainly due to high cell proliferation rates and inadequate vascular architecture. Thus, the presence of hypoxia impairs biological functions and increases the radiation resistance of tumors.

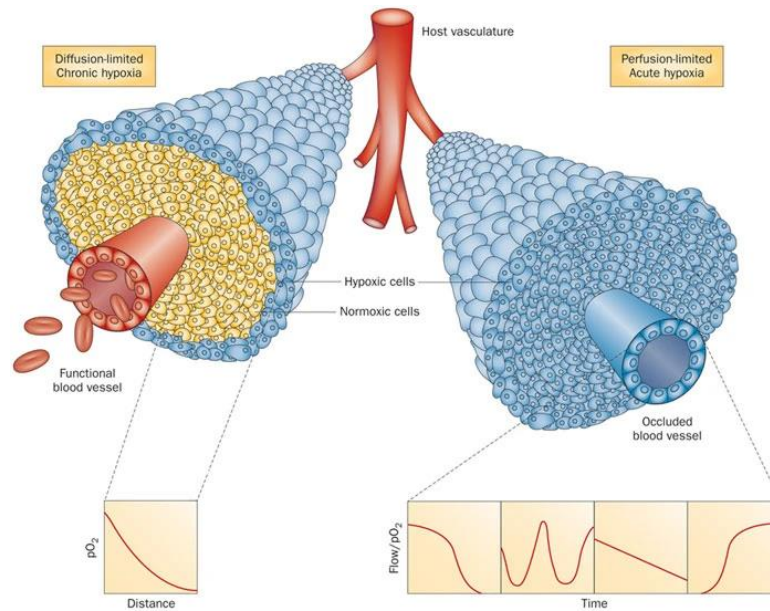


Figure 2.2. Comparison of the two forms of hypoxia, chronic (left) and acute hypoxia (right). The tumor cells grow in circles around the blood vessels that supply them with nutrients and oxygen. The left panel shows chronic hypoxia caused by a decrease in oxygen partial pressure with increasing distance from the capillary (see insert on the lower left [45]) so that the cells become chronically (constantly over time) hypoxic. The right panel represents a transiently occluded vessel that leads to an acute hypoxic state of all surrounding cells [46]. Causes of impaired blood flow in the vessel may include obstruction by blood cells or circulating tumor cells, spontaneous vasomotor activity upstream, or collapse of the vessels due to a high interstitial pressure [41]. The restricted blood flow can have different temporal profiles for the time window of an hour, as shown in the graphs below right. Reprinted with permission of Springer Nature from [41] who modified the illustration from [47]. Besides that, the reprint permission for the illustration in [47] was granted by Elsevier.

Additionally, this characteristic feature of tumors [48] affects important hallmarks of cancer [49] and was found to be an independent, adverse prognostic factor for patient survival [50]. Hypoxia can be separated into *anemic*, *acute*, and *chronic* (Figure 2.2) that can coexist in the same tissue sample and can be further differentiated into subgroups by considering pathogenetic and pathophysiologic mechanisms [51]. The causes of the different types of hypoxia are multifactorial and may lead to different biological changes and thus to different radiobiological responses [52], [53].

Forms of hypoxia

Anemic hypoxia occurs when tumor-associated or therapy-induced *anemia*, i.e., a decrease in the hemoglobin concentration in the blood, leads to a reduced oxygen transport capacity of the blood [48], [54], [55].

Acute (perfusion-limited) hypoxia was first described by Brown in 1979 [56] and experimentally verified by Chaplin et al in 1986 [57]. This form of hypoxia, shown in Figure 2.2 on the right, is caused by temporary perfusion changes in the capillaries in the time frame of minutes to hours [56]. This is attributable, for example, to vascular occlusion or reversed blood flow.

Two subtypes may be distinguished:

- (a) *Ischemic hypoxia* is caused by inadequate blood flow to tissues [58], e.g., circulatory disturbances or temporally constant decreased blood flow.
- (b) *Hypoxemic hypoxia* is induced by a decreased oxygen concentration within the capillaries, which may be caused by fewer erythrocytes in the blood plasma.

Chronic (diffusion-limited) hypoxia was first described by Thomlinson and Gray in 1955 [59] and occurs on time scales of several days [60]. Oxygen that diffuses out of the capillary is consumed by the surrounding tissue, resulting in a decrease of oxygen tension with increasing distance to the capillary (see the left panel of Figure 2.2). Since the vascular architecture is persistent over time, this situation is called chronic hypoxia. Chronic hypoxia may improve by neoangiogenesis, but the rapidly developing neovascular system is usually chaotic and primitive [41].

Hypoxic distances

Cells depicted as “normoxic” in Figure 2.2 may already be hypoxic to some degree although they are not classified as radiologically hypoxic [41]. At distances of 100 – 180 μm to the nearest blood vessel, a severe oxygen deficiency occurs [41], resulting in an acute oxygen absence (*anoxia*) for the most remote cells. Other authors estimate this oxygen diffusion distance to be only 70 μm at the arterial end and subsequently even less at the venous end of the capillary [61, p. 176]. Between the well-oxygenated and anoxic cell layers is a layer of hypoxic cells, which is about one to two cell layers thick. This moderately hypoxic layer contains cells that have still sufficient oxygen available to be viable but the layer already exhibits increased radioresistance compared to well-oxygenated tissue [61, p. 176].

Necrosis

In case that not only the oxygen supply is restricted, but also the supply with other metabolites, such as glucose which has about twice the diffusion distance of oxygen, the cells become *necrotic*. Therefore, tumors that reach a certain size typically develop a necrotic core.

2.1.3. Radiation response of tumors

For a successful radiation therapy of tumors, it is necessary to induce the death of all clonogenic tumor cells, i.e., all cells capable of cell division. The probability of cell damage depends on various properties of the radiation as well as those of the cells. Important properties of the radiation are, for example, radiation type, total dose, spatio-temporal pattern of dose delivery (dose distribution, dose rate, and fractionation [62]). As for tumor cells, the most important factors are intrinsic radiosensitivity, cell cycle phase, and TME factors.

The main targets of irradiation are DNA molecules since DNA damage has the most far-reaching consequences, e.g., loss of clonogenicity, cell function, ultimately leading to cell death. In particular, (clustered) double-strand breaks in the DNA helix represent the most important lesions [63] because they are more difficult to repair than single-strand breaks.

Survival fraction

The survival probability of cells is described by the *survival fraction* (SF) as

$$SF = \frac{N}{N_0}, \quad (2.11)$$

which describes the ratio of the number of surviving cells after irradiation N to the initial number of cells N_0 . The SF may be measured in vitro using the so-called *clonogenic survival assay*. In this assay, the survival of individual cells is determined by their ability to proliferate and form a visible number of cells [64].

Cell survival curves

The measurement of SF at different doses in vitro leads to so-called *survival curves*. These survival curves can be used to quantify the influence of physical treatment parameters and biological factors on the radiation response. They typically exhibit a linear-quadratic shape on a semi-log scale as shown in Figure 2.3 a).

An empirical description of survival curves is given by the *linear-quadratic model* (LQM) introduced by Chadwick and Leenhouts in 1973 [63]. The LQM is a second-order polynomial fit of the dose-dependent survival fraction:

$$SF(D) = \exp [-(\alpha D + \beta D^2)]. \quad (2.12)$$

In the LQM, the SF is characterized by the dose levels D and the tissue-specific parameters α and β , which describe the intrinsic radiosensitivity of the cells and are expressed in units of Gy^{-1} and Gy^{-2} , respectively. The parameters α and β are both used to describe the dose-dependence of the radiation response and are combined in the ratio α/β (unit: Gy), which describes the repair capacity of the cells. Low α/β ratios of 1.5 – 5 Gy are typically found for late-responding normal tissues (e.g., spinal cord, heart), whereas values between 6 – 14 Gy occur for acute reacting normal tissues (e.g., colon, skin, mucosa) and most tumors [65].

The higher intrinsic radiosensitivity of tumors compared to most normal tissues predominantly leads to radiation-induced sterilization of tumor cells and is thus the reason for the applicability of radiotherapy. The LQM has been successfully used to fit the radiation response for different fractionation schemes *in vitro* [66], and in combination with more advanced models, also *in vivo* for a wide dose per fraction range from 2 to 10 Gy [66]. Moreover, it still provides acceptable results up to 15 to 18 Gy based on animal results [67].

Dose-response curves

Depending on the total dose received by the tumor, the binary *in vivo* response is either “controlled” or “not controlled”. Clinically, the most relevant endpoints are local tumor control, disease-free survival, and complication in normal tissue. For *local control*, it is assumed that all clonogenic tumor cells have been killed at the site of the primary tumor. This is, e.g., clinically verified by the absence of further tumor growth in diagnostic images.

In vivo, the radiation response is represented by dose-response curves (DRC). DRCs are determined by measuring *tumor control probability* (TCP) and *normal tissue complication probability* (NTCP) for different dose levels, as shown in Figure 2.3 b). TCP and NTCP describe the probability that a given dose will cause tumor control (TCP) or complication in an organ or structure (NTCP), in each case taking into account the specific properties of the biological cells [68], [69].

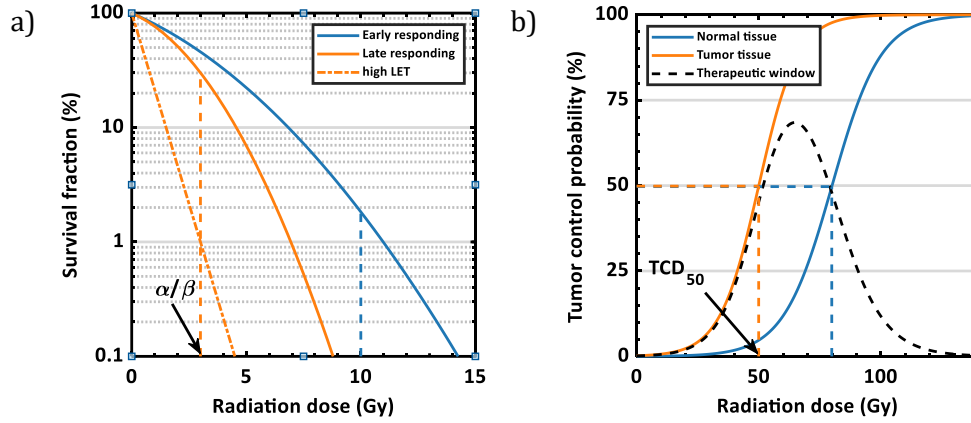


Figure 2.3. Types of survival curves. Panel a) shows the survival curves for cells with low repair capacity (orange solid curve, $\alpha/\beta = 10$ Gy) and high repair capacity (blue solid curve, $\alpha/\beta = 2$ Gy) for standard X-ray treatments. The two curves correspond to early and late responding normal tissues, respectively. The vertical dashed lines indicate the doses equal to α/β , where the linear (αd) and the quadratic (βd^2) term contribute equally to cell kill. Unlike the first two curves, the high-LET curve is much steeper and has no "shoulder", i.e., suppressed repair. Panel b) shows the dose-response curves of tumor and normal tissue. Successful radiation treatments require lower dose levels for tumor control compared to those leading to side effects in normal tissue. The area between the two curves is referred to as the *therapeutic window*, and treatment doses are chosen to maximize the tumor control probability (TCP) while minimizing the normal tissue complication probability (NTCP).

Experimentally, the TCP is usually determined by fitting a sigmoid curve to the incidence rates, i.e., the fractions of individuals who exhibit tumor controls at different dose levels. A commonly used TCP model is the *logistic dose-response model* as used in [70],

$$TCP(D) = \frac{\exp[b_0 + b_1 D]}{1 + \exp[b_0 + b_1 D]}, \quad (2.13)$$

which calculates the TCP for the total dose D with the fit parameters b_0 and b_1 . The parameter TCD_{50} is useful for comparing TCP curves as it indicates the total dose required to control 50 % of all irradiated tumors (see Figure 2.3 b)). It can be derived from Equation (2.13) as

$$TCD_{50} = -\frac{b_0}{b_1} \quad (2.14)$$

and describes the position of the TCP curve.

Oxygen enhancement ratio

Molecular oxygen is one of the most effective radiosensitizers, which means that the amount of oxygen present in a cell positively affects the biological effectiveness of the irradiation in tumor cells. This increased effectiveness is generally explained by the *oxygen fixation hypothesis*, which postulates that DNA damage caused by radicals is permanently fixed by molecular oxygen and thus cannot be repaired [71].

The radio-sensitizing effect of oxygen was experimentally demonstrated by Petry in 1923 [72] and was later transferred into a quantitative mathematical formalism by Alper and Howard-Flanders in 1956 [73]. Alper and Howard-Flanders defined the *oxygen enhancement ratio* (OER),

$$OER = \frac{D_{hypoxia}}{D_{oxic}} \Big|_{iso-survival}, \quad (2.15)$$

as the ratio of doses required to obtain the same biological effect, i.e., the same cell survival, under hypoxic ($D_{hypoxia}$) and oxic (D_{oxic}) conditions. The OER shows a hyperbolic dependence on the oxygen partial pressure (see Figure 2.4 a)) and can be phenomenologically described as in [73]:

$$OER(p_{O_2}) = \frac{mp_{O_2} + k}{p_{O_2} + k}. \quad (2.16)$$

The parameter m indicates the maximum OER and k describes the oxygen tension at which the OER reaches 50 % of its maximum value (see Figure 2.4 a)). The maximum OER of *in vitro* experiments usually ranges between 2.5 and 3, highlighting the importance of the oxygenation status for treatment planning.

Equation (2.16) can be used to also account for the sensitizing effect of oxygen in the LQM [74]:

$$SF(d, OER) = \exp[-(\alpha_{hyp}d \cdot OER + \beta_{hyp}d^2 \cdot OER^2)]. \quad (2.17)$$

The parameters α_{hyp} and β_{hyp} now refer to completely hypoxic conditions, (i.e., $OER = 1$). Figure 2.4 b) displays the influence of the oxygen status on the cell survival curves, whereas the radiation sensitizing effect of oxygen is usually considered to be dose-independent.

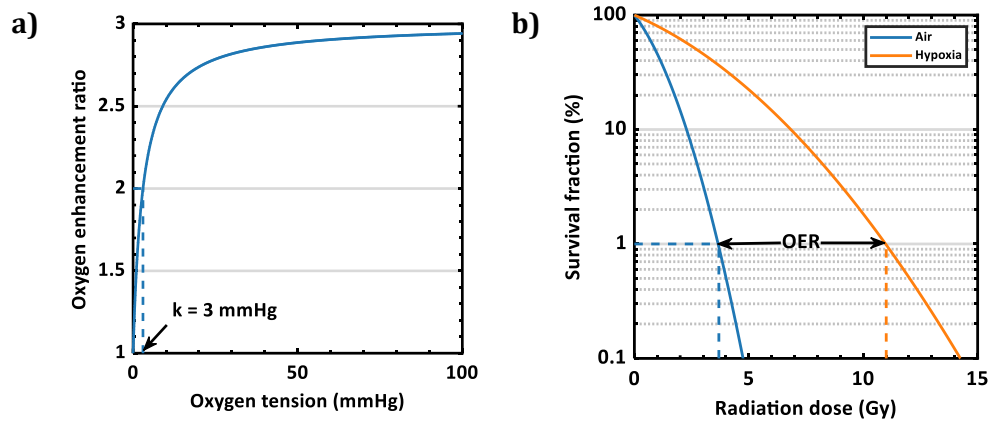


Figure 2.4. The oxygen enhancement ratio. a) Dependence of the radiosensitivity on the oxygen tension ($k = 3$ mmHg and $m = 3$ as in [61]). Most of the change in OER occurs below 30 mmHg [75]. The OER value of two, which lays in the middle between anoxia (OER = 1) and full oxygenation (OER = 3) occurs at an oxygen tension of 3 mmHg (i.e. an oxygen concentration of 0.5 %) [61]. b) Survival curves for the two extreme conditions of completely oxic and hypoxic cells with an indication of the OER for these conditions.

Relative biological effectiveness

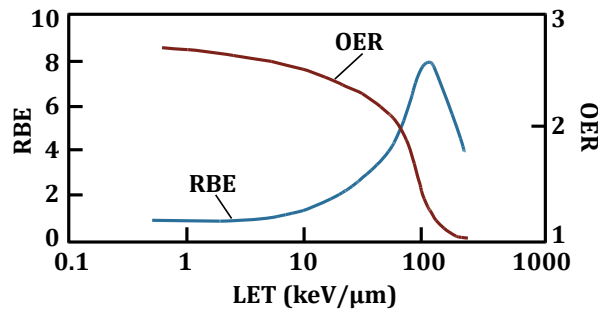


Figure 2.5. Dependence of relative biological effectiveness (RBE) and oxygen enhancement ratio (OER) on the linear energy transfer (LET) for human T1 kidney cells irradiated with various naturally occurring α -particles or with deuterons accelerated in the Hammersmith cyclotron. At a LET of 100 keV/ μ m, a rapid increase in RBE and drop of the OER occur. Adapted from [61] with permission from Wolters Kluwer Health, Inc, who adapted the figure from [76]. Besides that, the reprint permission for the illustration in [76] was granted by the IAEA.

The *relative biological effectiveness* (RBE) describes the ratio of the absorbed dose D_B of a certain type of radiation to the absorbed dose D_A of a reference radiation leading to the same biological effect:

$$RBE = \frac{D_A}{D_B} \Big|_{iso-effective} . \quad (2.18)$$

Figure 2.5 demonstrates that both the particle type and its energy play a role in the RBE calculation in addition to the type of target tissue.

Fractionated radiotherapy

Radiotherapeutic treatments are usually delivered in multiple daily fractions of typically 1.8 to 2 Gy for three to seven weeks, resulting in a total dose of 40 – 70 Gy [77, p. 461]. The breaks between individual fractions allow for the repair of sublethal damages, which requires 8 -10 hours for complete repair [77, p. 455]. Repair is more pronounced in normal tissue than in tumors, i.e., normal tissue benefits more from fractionation. Consequently, the therapeutic window (Figure 2.3 b)) can be widened by increasing the dose to the tumor (leading to higher TCP at the same NTCP) or decreasing the dose to the normal tissue (leading to lower NTCP at the same TCP).

2.2. Modeling of the tumor oxygenation

Bio-mathematical models are useful to understand the complex development of tumors, as the modeling allows separate analysis of the individual processes and identification of key biological parameters. This thesis focuses on the oxygen transport within tumors, which is modeled by the *diffusion-reaction equation* (DRE), a second-order partial differential equation. In the following, the fundamentals of this equation are presented and its implementation in the *tumor oxygenation model* (TOM). The last part of this section introduces the parameters that characterize the oxygen histograms generated by the oxygenation model.

2.2.1. Partial differential equations for oxygen transport

General transport equation

Oxygen transport exhibits a spatio-temporal dependence described by the *general transport equation*. This equation can be written for a general intensive variable ϕ , which may be identified as the oxygen concentration as

$$\frac{\partial(\rho\phi)}{\partial t} + \nabla(\rho\phi\mathbf{u}) = \nabla \cdot (D\nabla\phi) + S(\phi), \quad (2.19)$$

where ρ is the density and $\mathbf{u}$ the velocity of oxygen molecules, and D the diffusion coefficient [78, pp. 24–25]. In other contexts, ϕ can be e.g., the concentration for mass transfer calculations or the temperature for heat transfer problems. Equation (2.19) consists of four transport processes: The *rate of change* and the *convective* terms for a volume element on the left side and the *diffusive* and the *source* terms on the right side.

The *convection* term describes the flow of oxygen with the velocity $\mathbf{u}$ out of a volume element. Convective currents in the interstitial compartment are expected to contribute to only 0.5 – 1 % of the plasma flow (including molecular oxygen) in normal tis-

sues [60], whereas in tumors they may be as high as 15 % and thus cannot be neglected [54]. However, the convective transport of oxygen in tumors has not yet been successfully modeled [60] and is therefore also neglected in this thesis.

Diffusion-reaction equation

Replacing the general intensive quantity ϕ in Equation (2.19) by the molar oxygen concentration c and neglecting convection, the *diffusion-reaction equation* (DRE) is obtained. Assuming an isotropic diffusion coefficient D , the DRE can be written as

$$\frac{\partial c}{\partial t} = D\Delta c + S(c). \quad (2.20)$$

Equation (2.20) belongs to a group of parabolic equations, which are typically used for time-dependent diffusion-dominated processes [78, p. 28]. The isotropic diffusion coefficient D was first characterized by Einstein in 1905 [79], who studied the Brownian motion of particles in a suspension using the random walk model and derived the *Einstein-Smoluchowski equation*:

$$\langle x^2 \rangle = 2Dt. \quad (2.21)$$

Equation (2.21) describes the mean squared distance $\langle x^2 \rangle$ that particles are displaced in a Gaussian diffusion process (resulting from Boltzmann statistics) as a function of time. Water, and thus approximately for body tissue, exhibits for example a displacement of the water molecules of about $4 \mu\text{m}^2$ in 1 ms for the diffusion coefficient D_{water} of $2 \mu\text{m}^2/\text{s}$.

With the assumption that the oxygen concentration follows the general gas equation for ideal gases

$$pV = nRT, \quad (2.22)$$

the oxygen partial pressure p can be considered proportional to the concentration c :

$$c = \frac{n}{V} \stackrel{\text{Equation (2.22)}}{=} \frac{p}{RT}. \quad (2.23)$$

Thereby V denotes the volume, n the chemical amount, T the temperature, and R the universal gas constant. Thus, Equation (2.20) can be rewritten as

$$\frac{\partial p}{\partial t} = D\Delta p + S(p). \quad (2.24)$$

The spatio-temporally dependent oxygen partial pressure $p = p_{\text{O}_2}(\mathbf{x}, t)$ is governed by a diffusion and source/reaction term. Assuming steady-state conditions, i.e., $\partial p / \partial t = 0$, Equation (2.24) further simplifies to

$$0 = D\Delta p + S(p). \quad (2.25)$$

Source/reaction term

The source/reaction term $S(p)$ in Equation (2.25) describes both the oxygen supply by the capillaries and the oxygen consumption by the surrounding tissue. The oxygen partial pressure in all capillaries is assumed to be temporally constant

$$p_{Cap}(x, t) = \text{const. } \forall t, \quad (2.26)$$

with a maximum value of 40 mmHg as in [27], [43], [80]–[85]. This is a typical value for venous blood [85], from which, however, the values in tumors may differ significantly due to their chaotic vessel architecture [82]. The oxygen consumption is modeled by the hyperbolic Michaelis-Menten kinetics as in [27], [43], [84], [86], [87]:

$$g_{Tissue}(p) = g_{max} \frac{p}{p + k}. \quad (2.27)$$

The constants g_{max} and k describe the maximum oxygen consumption rate and the oxygen tension, at which a half-maximum consumption occurs, respectively. Figure 2.6 illustrates that the oxygen consumption increases rapidly with oxygen availability up to 30 mmHg and saturates at higher values.

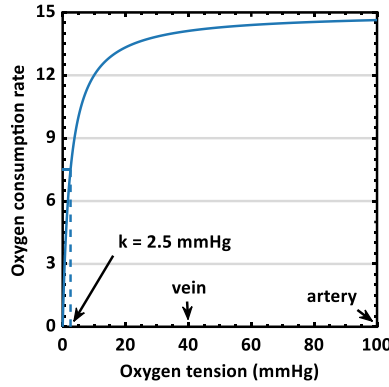


Figure 2.6. Oxygen consumption as a function of the oxygen partial pressure. The half-maximum consumption rate of 7.5 mmHg/s occurs at the $p_{O_2} = 2.5$ mmHg for a selected $k = 2.5$ mmHg as in [80].

Discretization of the diffusion term

One of the following three numerical methods is commonly used to discretize the diffusion term $D\Delta p$ of the DRE: The *finite difference method* (FDM), the *finite element method* (FEM), or the *finite volume method* (FVM). [88]. In this thesis, the *particle strength exchange method* (PSEM), a finite volume approach, and the FDM were used

as described below. The difficulty in implementing the FEM for complex vascular geometries was a reason for not using this method although shorter calculation times could be achieved.

Particle strength exchange method (PSEM)

The deterministic PSEM [89], [90] utilizes a mass-conserving integral to discretize the Laplace operator,

$$\Delta_\varepsilon p(\mathbf{x}) = \frac{1}{\varepsilon^2} \int_{\mathbb{R}^n} (p(\mathbf{y}) - p(\mathbf{x})) \cdot \eta_\varepsilon(\mathbf{y} - \mathbf{x}) d^n y, \quad (2.28)$$

and thus the “exchange strength” between two particles at the positions $\mathbf{x}$ and $\mathbf{y}$ is estimated, i.e., the exchange of an intensive quantity, such as the oxygen tension p . The first term on the right of Equation (2.28) is the p_{O_2} difference between neighboring voxels, and the second a Gaussian kernel function for the p_{O_2} gradient [27]. The constants ε and n describe the interaction distance between the grid points and the physical dimension, respectively. Equation (2.28) can be discretized for a point grid as

$$\Delta_\varepsilon p(\mathbf{x}) = \frac{1}{\varepsilon^2} \sum_{i=1}^N (V_j p(\mathbf{x}_j) - V_i p(\mathbf{x}_i)) \cdot \eta_\varepsilon(\mathbf{x}_j - \mathbf{x}_i), \quad (2.29)$$

where V_i is the volume of the i th grid point. The multi-dimensional Gaussian kernel

$$\eta_\varepsilon(\mathbf{x}_j - \mathbf{x}_i) = \frac{1}{(\sqrt{\pi}\varepsilon)^d} \exp \left[-\frac{\|\mathbf{x}_j - \mathbf{x}_i\|^2}{\varepsilon^2} \right], \quad (2.30)$$

was used, with the standard deviation σ substituted by the interaction distance $\varepsilon = \sqrt{2}\sigma$.

Finite difference method (FDM)

The FDM uses the *second-order differential quotient* to approximate the Laplace operator. The one-dimensional central difference quotient

$$\begin{aligned} \Delta p(x) &= \frac{\Delta^2 p}{\Delta x^2} = \frac{p(x_{i+1}) - 2p(x_i) + p(x_{i-1}))}{\Delta x^2} \\ &= \frac{p(x + \Delta x) - 2p(x) + p(x - \Delta x))}{\Delta x^2} \end{aligned} \quad (2.31)$$

is calculated for a point x from the difference of the partial pressures of its two neighboring grid points $x - \Delta x$ and $x + \Delta x$ in a distance, i.e., voxel size, Δx . Equation (2.31) can be easily extended to multiple dimensions, as in this work for the 2D and 3D cases.

Boundary conditions

The simulation volume of numerical simulations is limited to a finite size, mainly due to constraints in computing power and memory size. Thus, the volume boundaries, play a crucial role in computational accuracy and efficiency and must satisfy certain *boundary conditions* (BC). Common BC are the *Dirichlet*, *von Neumann*, and the *periodic* BC, and the appropriate one must be selected depending on the simulated problem.

The *Dirichlet (first-type) BC* specifies a fixed value of the solution of the differential equation on the domain boundary. This could, i.e., be oxygen tension on the volume boundary for oxygen diffusion simulations. The Dirichlet BC is used in this work to fix the p_{O_2} within the capillaries to a constant value.

The *von Neumann (second type) BC* specifies the value of the first derivative perpendicular to the domain boundary, which in our case would be the oxygen flux across the boundary.

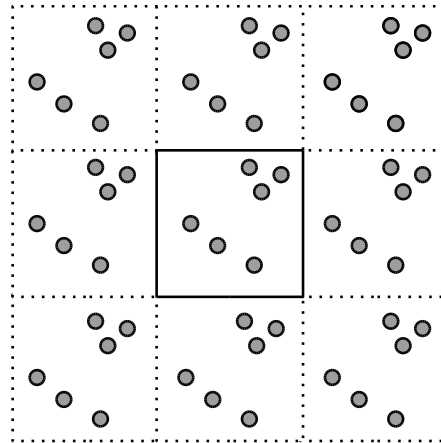


Figure 2.7. Schematic representation of the periodic boundary conditions in 2D. The square simulation domain in the center with five circular capillary cross-sections continues periodically across the domain boundaries by translations.

The *periodic BC* periodically extends the simulation volume at the edge by defining grid points on one side of the simulation volume as adjacent to those on the opposite side (see Figure 2.7). In this work, the periodic BC is used for the grid points at the volume boundaries to account for the oxygen contributions from outside the simulated volume. This allows reducing the size of the simulated volume since there is no need to exclude a safety margin in proximity to the volume borders to avoid boundary effects. This makes it possible to reduce the size of the simulated volume of the randomly distributed synthetic capillaries since it is not necessary to exclude a safety volume at the volume borders to avoid edge effects.

2.2.2. Numerical solution methods

Due to complex vessel architectures, an exact solution of a partial differential equation is often not possible. Therefore, numerical methods are necessary to generate an approximation to the solution. These methods can be divided into *implicit* and *explicit* algorithms.

In the *explicit* algorithm, the state of a time-dependent system $Y(t)$ is calculated in a forward-marching procedure as

$$Y_{t+1} = Y_t + f(Y_t). \quad (2.32)$$

Thus, only the results from the previous time step Y_t are used to calculate those of the following step Y_{t+1} , while the function $f(Y_t)$ contains all physical processes contributing to the evolution of the system.

In contrast, the *implicit* scheme uses the information of the time step Y_{t+1} on both sides of the equation:

$$Y_{t+1} = Y_t + f(Y_{t+1}). \quad (2.33)$$

This leads to a more complex implementation but does not require an additional stability condition for the solution as in the case of the explicit method [78, pp. 243–249]. Besides that, the implicit method is generally more robust and reduces the computational effort. The numerical accuracy of both methods can be increased with smaller step sizes.

2.2.3. Tumor oxygenation model (TOM)

Features of the initial *TOM 2013* version

Espinoza et al. [27] developed the framework *tumor oxygenation model* (TOM) to simulate oxygen distributions in tumors. The TOM is a voxel-based model written in *MATLAB Version 7.13 (R2011b)* [91] that solves the steady-state DRE (Equation (2.25)) either on a 2D or 3D simulation grid. Table 2.1 shows the standard parameters for TOM simulations.

Table 2.1. Default parameters for simulations with the *TOM 2013* version. Values were taken from [27].

Name	Symbol	Value	References
<i>Biological parameters</i>			
Capillary radius	r	10 μm	[92]–[94]
Diffusion coefficient of molecular oxygen (at 37 °C)	D	$2 \cdot 10^{-9} \text{m}^2/\text{s}$	[80], [95]
Maximum oxygen consumption rate (tissue)	g_{max}	15 mmHg/s	[43], [59], [80], [96]
Oxygen tension* (capillaries)	p_{cap}	40 mmHg	[59], [94]
p_{O_2} at half maximum consumption rate	k	2.5 mmHg	[43], [80], [96]
Vascular fraction	VF	1 – 7 %	
<i>Simulation parameters</i>			
Capillary architecture		parallel linear	
Dimensions	d	2	
Interaction distance	ε	22 μm	
Numerical method	PSEM	Particle strength exchange	[89], [90]
Steady-state condition	tol	400 iterations	
Time step	Δt	0.07 s	
Verlet list cutoff	l_{cut}	80 μm	
Voxel size (i.e., grid spacing, mesh width)	dx	20 μm	

*An oxygen tension of 40 mmHg (= 5.33 Pa) means that 5 % of the total gas mixture in the capillaries is oxygen. The value of 40 mmHg is found, for example, in the venous system and also in tumors since the latter are often supplied from the venous side of the vasculature [97].

The TOM simulation grid is composed of two tissue types, “capillary cells”, and “tumor cells”. Oxygen originates from the capillary centers and diffuses into the tissue, where it is consumed with a concentration-dependent rate (Equation (2.27)). Capillaries are approximated as linear parallel lines oriented parallel to one of the Cartesian axes that contain a fixed oxygen tension (Dirichlet BC).

The TOM uses the explicit *forward Euler* method to iteratively solve the DRE [98], [99, p. 35], [100, p. 45]. In this scheme, first described by L. Euler in 1768 [98], each successive time step p_{t+1} is derived from the previous step p_t as

$$p_{t+1} = p_t + dt \cdot f(p). \quad (2.34)$$

$f(p)$ contains all relevant physical processes specified in the DRE (oxygen supply, diffusion, and consumption), dt is the time increment between two steps. The simulation is terminated after 400 iterations. This termination condition, described by the integer tol (see Table 2.1) is used to finish the simulation when the steady state, i.e., the

equilibrium between oxygen supply and consumption, is approximately reached. The resulting oxygen tensions of all tumor voxels are condensed into oxygen histograms as exemplified in Figure 2.9 in Section 2.2.4.

Key findings of Espinoza et al.

Espinoza et al. [27] have shown, that a 2D grid with parallel linear capillaries leads to similar oxygen histograms as a 3D grid with parallel and orthogonal linear capillaries. In both cases randomly distributed artificial linear capillaries were used as simulation input. Besides that, completely randomly placed vessels led to the most similar synthetic IVD histograms to experimental IVD histograms for murine carcinoma (data from [92]) compared to more regularly arranged capillaries.

Updates in the *TOM 2015* version

The TOM was updated in 2015 by A. Gago and I. Espinoza (unpublished), who made two major modifications: (i) Replacing the numerical algorithm for solving the diffusion term from the PSEM to the FDM. (ii) Changing the termination condition from the maximum number of 400 iterations (parameter *tol*) to termination if the *maximum absolute p_{O_2} difference* of all grid points between two consecutive time steps dt is smaller than 10^{-5} . For a definition of this termination condition, see Section 2.2.4.

Updates in the *TOM 2021* version

In this thesis, a complete revision of the code used in [27] was performed to increase the speed of TOM simulations, allowing for larger simulation volumes and smaller voxel sizes. The following two sections describe these steps which were implemented using *MATLAB Version 9.8 (R2020a)* [101].

Reduced computation times through vectorization and Verlet lists

The numerical implementation became computationally more efficient with three changes:

- (i) The code was vectorized, i.e., rewritten in vector and matrix form. This improved MATLAB's performance, as it is optimized for vector and matrix operations.
- (ii) The *Verlet* lists, implemented in the TOM 2013 version, were originally developed to accelerate simulations, e.g., by restricting the interactions between grid points to local interactions with the nearest neighbors below a critical radius. It turned out, however, that for sufficiently small grid sizes, it is more effective to calculate all interactions in one step instead. Since all interactions are limited to a local neighborhood, a large simulation grid can be replaced by smaller sub-grids and thus the Verlet lists could be omitted.

- (iii) The diffusion function was accelerated using the *circshift* function of MATLAB [101], which shifts an entire array in the specified direction. This replaced the loop to calculate the oxygen tension difference between a voxel and its neighbors. The *circshift* function was also implemented for the periodic BC, which are used at the volume boundaries.

In addition to these changes, the code structure was adapted to treat all simulation parameters as external input parameters. For the standard grids, used e.g. in Espinoza et al. [27], the revision resulted in an acceleration for the 2D capillary grids by a factor of 100 with a total runtime of 80 ms per simulation (average of 100 simulations). The acceleration for the 3D cases was 270-fold, resulting in a run time of 19 s per simulation (average of 10 simulations). These simulations were performed on a personal computer equipped with an Intel® Xeon® CPU E5 quad-core processor with 3.50 GHz and 64 GB RAM that was used for all simulations in this thesis.

Reduced machine precision increased performance

For data requiring large matrix sizes, such as high-resolution images, one usually switches from *double-precision* to *single-precision* floating-point numbers (Table 2.2). This reduces the file sizes to 50 % per dimension and accelerates the calculations. However, this also results in a loss of accuracy, since the machine precision ε , the smallest difference to distinguish two adjacent numbers, is also reduced by a factor of 10^8 (Table 2.2). The reduced accuracy had no significant effect on the resulting oxygen distributions.

Table 2.2. Machine precision for different types of floating-point numbers.

Precision	Bytes per number	Machine precision ε
Single	32	$1.2 \cdot 10^{-07}$
Double	64	$2.2 \cdot 10^{-16}$

For simulation volumes, as in Espinoza et al. [27], the change from double to single precision led to opposite effects in terms of simulation time for 3D and 2D simulation grids: A minor acceleration of 7 % was visible for the 3D case that uses 125 000 grid points in the TOM 2013 version. However, the calculation time increased by 36 % for 2D simulations with 2 500 grid points, caused by the computational overhead in converting the floating-point numbers from double to single. The conversion is necessary because MATLAB's standard format is double, so one must convert the matrices to single precision after their allocation.

This overhead (in the order of milliseconds per simulation) becomes less relevant with larger simulation grid sizes or smaller voxel sizes, justifying the global implementation of single precision for subsequent simulations that use smaller voxel sizes.

Also, both accuracies showed comparable accuracy for the simulation grids mentioned (data not shown).

Implemented numerical algorithms

Both FDM and PSEM have been included in the main function of the TOM to allow greater flexibility in the choice of parameters.

Decoupling the number of capillaries from the voxel size

TOM 2013 method for calculating the number of capillaries

A standard simulation setup for the TOM 2013 simulations included linear capillaries and voxel sizes of 20 μm . For a graphical representation of the simulated oxygen distributions as in Figure 3.4 a), one voxel is typically assigned to each grid point, with the grid points at the voxel centers (Figure 3.5).

To create a capillary distribution for a simulation volume V_{total} , the vascular fraction (VF) is used to calculate the number of capillaries N_{total} . Assuming an equal volume V_{cap} for each capillary, Equation (2.8) can be rearranged to compute the number of capillaries as

$$N_{\text{total}} = VF \frac{V_{\text{total}}}{V_{\text{cap}}}. \quad (2.35)$$

In the TOM 2013 version, the cross-section of a linear capillary filled one voxel in the simulation grid. To account for the spatial extend of the capillary with a diameter d of 20 μm , the point-shaped cross-section was treated as one grid voxel with a side length a of 20 μm . Thus, the capillary cross-section filled square area $A_{\text{sq}} = a^2 = 400 \mu\text{m}^2$. This area was then used to calculate the capillary volume with Equation (2.35), whereas the V_{cap} is calculated by the capillary area multiplied by its length.

Using a smaller voxel size of, for example, 10 μm instead of 20 μm for the same simulation grid, the capillary cross-section would be approximated by $A_{\text{sq}} = (10 \mu\text{m})^2 = 100 \mu\text{m}^2$ instead. Consequently, the calculated number of vessels would be four times higher for the four times reduced area according to Equation (2.35). This change in the number of capillaries, i.e., the number of oxygen sources, directly correlates with tissue oxygenation. Hence, in this example, a change of voxel size for the linear capillaries affects the oxygenation directly.

This effect occurs for arbitrary capillary cross-sections when changing the voxel size. For example, an idealized capillary with a circular cross-section would occupy a certain number of grid points on the grid due to its spatial extension. The smaller the chosen voxel size, the better the approximation of the actual shape of the capillary, and a higher number of grid points is classified as a "capillary voxel". As a result, the

capillary volume would change with each modification of the voxel size, as was previously the case with the point-shaped capillary cross-sections. Thus, simulation volumes with different voxel sizes for the same vascular fraction would have a different number of capillaries.

TOM 2021 method for calculating the number of capillaries

To avoid the different number of capillaries dependent on the voxel size and thus, to make simulations for different resolutions and different capillary cross-sectional shapes more comparable, the calculation of the number of capillaries in the volume was rearranged in this work:

First, the volume of an idealized capillary with a circular cross-section is calculated, using the length of the capillary, the diameter d , and the cross-sectional area $A_{\text{ideal}} = \pi(d/2)^2$. The volume is then used to calculate the number of capillaries for the 2D case as

$$N_{\text{total}} = VF \cdot \frac{A_{\text{ideal}}}{A_{\text{total}}}. \quad (2.36)$$

Equation (2.36) can be easily extended for the 3D case by multiplying A_{circ} with the length of the capillaries and A_{total} with the length of the third grid dimension. Thus, the number of capillaries becomes independent of the space-filling of the capillaries and the shape of the capillary cross-sections.

Impact of this algorithm modification

The effect of this algorithm update would mean for the previously mentioned TOM 2013 parameters that the ideal circular area for a capillary diameter d of 20 μm would be $A_{\text{ideal}} = 314 \mu\text{m}^2$ and thus 27 % smaller than the square area A_{squ} of 400 μm^2 . A 27 % reduced capillary area would lead to a 27 % higher N_{total} and consequently, would shift the simulated oxygen distributions towards higher values. This effect is exemplified in Figure 2.8 for a baseline vascular fraction of 3.6 %. An increased vascular fraction by 27 % to a value of 5 % for the same simulation grid and thus a 27 % higher N_{total} leads to a significant decrease of the frequency in the lowest bin, i.e., a reduction of severe hypoxia.

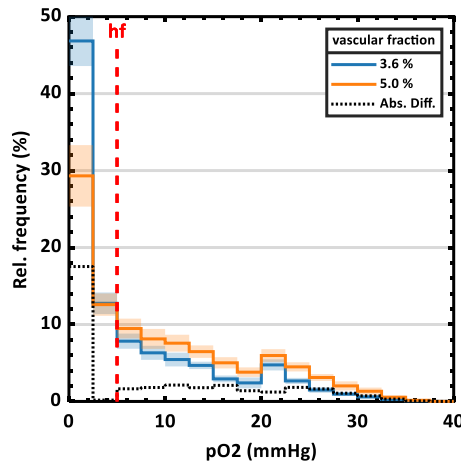


Figure 2.8. Impact of the algorithm change for calculating the number of capillaries in a simulation volume. The oxygen histograms show the means $\pm$ standard deviations of 20 independent simulations for 2D capillary grids with circular capillary cross-sections and capillary diameters of 20 μm . The quantitative comparison of the two curves shows a MAD of 2 %, a MaxAD of 17 %, an EMD of 8 %, and a DER of 1.08 at a 1 % SF. The DER means, that an 8 % higher dose would be required to reach the same 1 % survival fraction for the more hypoxic sample (blue curve) in a single dose treatment. For a parameter description see Section 2.2.4.

2.2.4. Parameters associated with the oxygen histograms

The simulated spatial oxygen tensions can be represented graphically as a 2D or 3D oxygen tension map to investigate the spatial correlation with the vessel distribution. A more condensed representation is possible with oxygen histograms with typically 16 bins, as shown in Figure 2.9. This representation is particularly advantageous for comparing different oxygen distributions from different simulations and for calculating the radiation response of the entire volume. Although the spatial information is lost when using the histograms, the oxygenation heterogeneity is still represented. By sampling the distribution, this heterogeneity can be considered when calculating the radiation response of the tumor. Besides that, parameters such as mean pO₂ and hypoxic fraction (Figure 2.9.) may be used to characterize tumor oxygenation and hypoxia, with the limitation that they provide less detailed information on the oxygenation than the whole histogram.

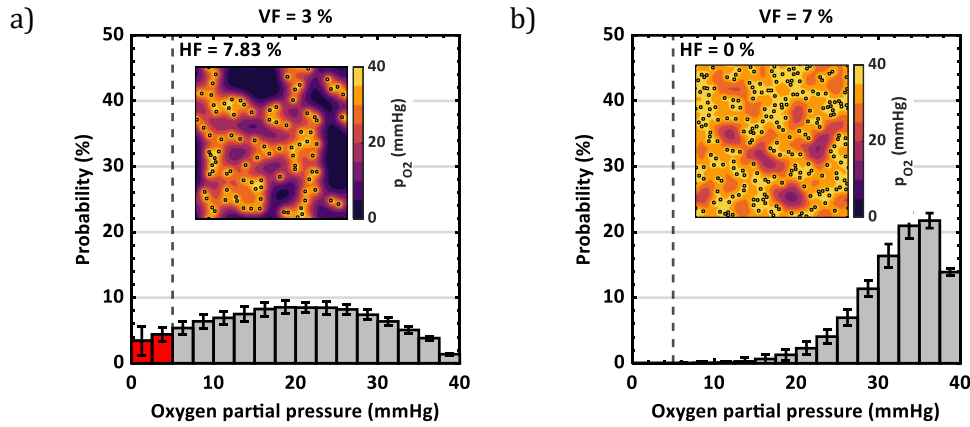


Figure 2.9. Spatial oxygen distributions and oxygen histograms for two different vascular fractions (VF) of a) 3 % and b) 7 %. The simulated 2D areas with capillaries perpendicular to the image plane had a size of 1 mm². The capillaries had a circular cross-section and a diameter of 20 μ m. The capillary borders are marked in black in the spatial oxygen distribution. The oxygen histograms show the means $\pm$ standard deviations of 20 independent simulations. The simulation yielded mean oxygen tensions of a) 20.3 and b) 33 mmHg and hypoxic fractions (HF) of 8 % and 0 % marked as red bins in the histograms.

Hypoxic fraction

In experiments, the *hypoxic fraction* (HF) is defined as the volume fraction with an oxygen partial pressure below an arbitrarily chosen threshold [27], [75, p. 332]. In this thesis, the threshold is set to 5 mmHg, which corresponds to the two lowest histogram bins in Figure 2.9. This threshold is supported *in vitro* by measurements of metabolic functions of cells. Gerweck et al. [102], for example, measured reduced *Adenosine triphosphate* (ATP) levels for an oxygen tension below 5 mmHg in Chinese hamster ovary cells. Moreover, the 5 mmHg threshold showed to be of significant prognostic value for disease-free and overall survival [103], [104].

A different hypoxia threshold is used when fluorescent makers are utilized. Fluorescent markers are commonly used to detect hypoxia at the cellular level in immunohistochemically (IHC) stained tissue sections. Hypoxia markers bind to specific molecules at oxygen tensions of 10 – 20 mmHg [48], e.g. in the case of the marker *pimonidazole*, to thiol-containing proteins that are specific for hypoxic cells [105]. Pimonidazole, which was used in this work, has been shown to be sensitive to p_{O_2} values below 10 mmHg in *in vitro* and *in vivo* studies for cervical carcinomas [106], [107] and proved to be a qualitative and quantitative marker for this type of carcinomas [105].

Quantities for comparing two oxygen histograms

In this work, three types of metrics were used to quantify the deviations between two oxygen histograms: Metrics with the suffix “deviation” were used to measure changes

in individual histogram bins. The *earth mover's distance* (EMD) on the other hand compares the similarity of the histogram shapes and the dose enhancement ratio provides an estimate of the radiobiological effect of histogram differences.

Quantities describing the probability differences

The *mean absolute deviation* between two probability histograms with the same number of bins n with the relative bin frequencies f_i and g_i was defined as

$$\text{MAD} = \frac{\sum_{i=1}^n |f_i - g_i|}{n}. \quad (2.37)$$

The corresponding *mean absolute percentage deviation* was then calculated as

$$\text{MAPD} = \frac{\sum_{i=1}^n \frac{|f_i - g_i|}{g_i}}{n}. \quad (2.38)$$

The *maximum absolute deviation*, useful for detecting outliers, was defined as

$$\text{MaxAD} = \max |f_i - g_i|, \quad (2.39)$$

and analogously the *maximum absolute percentage deviation* :

$$\text{MaxAPD} = \max \frac{|f_i - g_i|}{g_i}. \quad (2.40)$$

Earth mover's distance

The *earth mover's distance* (EMD) is a distance measure between two probability distributions [108] or more generally between two clusters. The EMD for two 1d distributions can be expressed as in [109] using the cumulative distribution functions $F(x)$ and $G(x)$ of the corresponding probability density functions f and g :

$$\text{EMD} = \int_{-\infty}^{\infty} |F_f(x) - G_g(x)| dx. \quad (2.41)$$

If the two distributions are probability distributions, the EMD is a true metric [108], also known in mathematics as the 1st Wasserstein distance [110].

The self-written MATLAB function for the EMD calculation used in this work was adapted for 1d probability histograms and normalized to the range 0 to 100 %. An EMD of 0 % means the histograms are identical, whereas 100 % means that the two histograms are maximally different, i.e., all entries of one histogram must be moved from the first to the last bin to achieve congruence of the two histograms.

Dose enhancement ratio

The *Dose enhancement ratio* (DER) was defined in this work as the ratio of two doses to achieve the same effect, i.e., equal cell survival under two different oxygenation conditions:

$$\text{DER} = \frac{D_{\text{oxy } 1}}{D_{\text{oxy } 2}} \Big|_{\text{isoeffect}} \quad (2.42)$$

Thus, the DER may be used to quantify the impact of the difference in oxygen distributions on the resulting change in the radiation response of the tumor. If *oxygenation condition 1* refers to complete hypoxia, the DER is equal to the OER (see Equation (2.15)). In the general situation, the oxygenation conditions may refer to different oxygen histograms. Then, the number of surviving tumor cells may be calculated using Equation (2.16) and (2.17) taking the p_{O_2} values as samples from the oxygen histograms.

In this work, the doses corresponding to a 1 % survival fraction for the DER calculation were determined with the unrestricted nonlinear minimization solver *fminsearch*, a Nelder-Mead Simplex method [111] of MATLAB's Optimization Toolbox [101], [112].

2.3. Experimental basics

2.3.1. Work distribution within the cooperation project

The experimental parts of this work were performed as a collaboration between the Heidelberg Institute for Radiooncology (HIRO) and the OncoRay (Dresden). The collaboration was established within the joint funding project "Characterization of vascular damage and hypoxia in experimental head and neck squamous cell carcinomas after irradiation with photons, protons, and carbon ions for the validation of biomathematical models" [113], funded by the National Center for Radiation Research in Oncology (NCRO).

The mouse models used in this work were acquired by J. Müller (OncoRay, Dresden, Germany) as part of his Ph.D. project [114] and shared for this work as part of the NCRO collaboration. In the collaboration, J. Müller performed the animal experiments, i.e., irradiation, imaging with computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound (US), as well as the tumor tissue extraction and tissue clearing. Besides that, he conducted the 2D imaging of the IHC stained histological sections and computed the raw data of the 2D sample analysis that was used in Section 3.2.1.

The rat tissues, i.e., the AT1 tumor and brain samples, were obtained by C. Glowa (German Cancer Research Center, Heidelberg), who also performed the animal handling,

as well as tumor and brain tissue collection. The subsequent tissue clearing was performed by J. Müller.

The 3D LSFM microscopy was performed for 2/3 of the specimens by J. Müller and 1/3 as part of this work. The following analysis of the 3D data and simulation of the oxygen distributions using TOM was performed as part of this work.

2.3.2. Tumor sample preparation

Tumor models

In this work, two established and well-characterized human tumor xenograft models [22], [51], [114], [115] of the *head and neck squamous cell carcinoma* (HNSCC) were used. Photon experiments revealed lower radiosensitivities based on the TCD_{50} for the SAS model compared to the UT-SCC-14 model [22]: The TCD_{50} values (with 95% confidence interval (CI)) were 44.2 (30; 53) Gy for the UT-SCC-14 model after 30 fractions of 2 Gy/fx and 99.1 (91; 108) Gy for the SAS model after 30 fractions of 1.8 Gy/fx. Furthermore, histological analyses indicated that the hypoxic volume for the SAS model remained constant under irradiation and no reoxygenation occurred, whereas the hypoxic volume in the UT-SCC-14 model decreased by approximately 50 % due to reoxygenation [22].

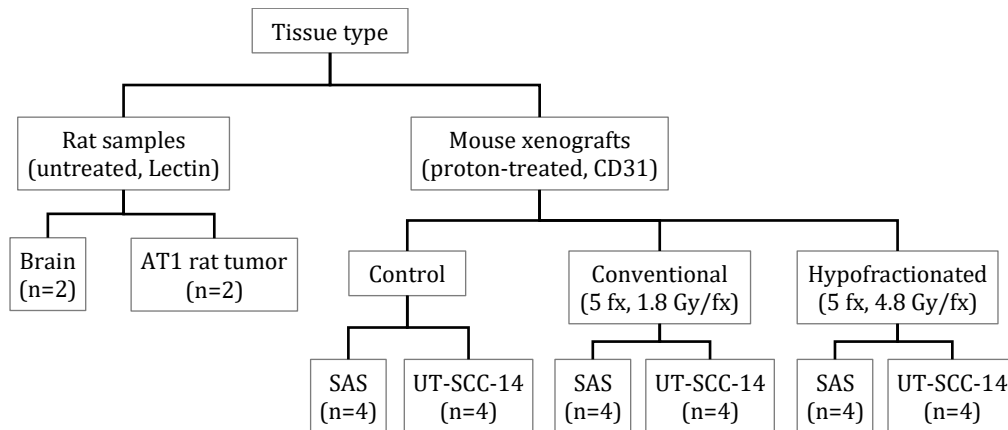


Figure 2.10. Tissue types used in this work, grouped by animal species and dose groups. n = number of animals used in this work for the vascular architecture characterization, fx = fraction, lectin = IF perfusion marker, CD31 = IF vessel marker for endothelial cells

In addition, two untreated rat tissue samples were used, as depicted in Figure 2.10: The established Dunning rat carcinoma subline R2327-AT1, which was characterized in [116], [117]. The AT1 tumor line exhibits poor differentiation and develops vol-

ume-dependent hypoxic areas due to the mainly tiny capillary structure [118]. Furthermore, two complete rat brains were used as reference normal tissue, which, unlike the chaotic tumor networks, have a highly regular vascular network, leading to better oxygenation and a lack of hypoxic regions.

Animal handling and preparation

The animal handling and preparation were performed by J. Müller as described in [114, pp. 67–68]. The following description closely follows the referenced work, which is based on the previously published experiments in [22], [115], and [119]:

Seven- to 14-week-old male and female NMRI (nu/nu) nude mice were obtained from pathogen-free animal breeding (Experimental Center and OncoRay, Faculty of Medicine, Technical University of Dresden, Germany) [115]. To suppress any residual immune response of the mice to the transplanted tissue, the mice were exposed to four Gray of X-ray radiation (200 kV, 1 Gy/min, 0.5 mm copper filtration) two to five days before tissue transplantation. The two HNSCC tumor lines SAS and UT-SCC-14 that exhibit a diverse radiation response, were used to create an artificial animal cohort.

The experiments were carried out as follows: Small pieces of tumor material were thawed from storage at -80°C and transplanted subcutaneously onto the backs of the first cohort of source animals with intraperitoneal (i.p.) injection of 120 mg/kg ketamine and 16 mg/kg xylazine. After sufficient tumor growth for volume doubling time (VDT) determination, tumors were excised and passaged to the backs of the second generation of source animals. After verification of correct VDT in this generation of tumors, viable tissue samples were collected and transplanted subcutaneously onto the hind legs of anesthetized mice.

The size of the tumors was measured twice a week with a caliper. When tumor diameters reached approximately seven millimeters, tumors were randomly assigned to an experimental group (control, conventional fractionation, or hypo-fractionation) if the respective VDTs were within the expected range. An additional animal was transplanted along with each cohort to obtain a VDT control and to verify the identity of the tumor model by microsatellite analysis and histological verification. All animals were sacrificed after completion of the planned experimental procedures or when the tumor burden of the animal exceeded a predefined threshold. VDT animals were sacrificed before tumor diameter reached a maximum diameter of 15 mm.

The animals were housed in Euro Standard Type III with up to five animals per cage, with a 12/12 h artificial daylight cycle and food and water *ad libitum*. The animal facility was approved and experiments were performed following the European Parliament and Council Directive (EU Directive 2010/63/EU) on the protection of animals

used for scientific purposes, the German Animal Welfare Ordinance, and the local ethics committee (approval TVV 2016-78, DD24-5131/35482 and TVV2018-50, DD24.1-5131/449/52, Dresden, Germany).

Fractionated irradiations

The animal irradiations were performed by J. Müller as described in [114, pp. 68–69]. The following description closely follows the referenced work, except for the exclusion of the photon arm, of which no animals were utilized in this work.

Table 2.3. Ratio of the applied total RBE-weighted doses and TCD₅₀ for the normal and hypofractionated UT-SCC-14 and SAS tumor models.

Fraction.	D_{tot} in Gy	$D_{\text{tot,RBE}}$ in Gy (RBE)	Tumor model	TCD _{50,phot} * in Gy (95 % CI)	$D_{\text{tot,RBE}}/\text{TCD}_{50}$ in % (95 % CI)
Normal	9	9.9	UT-SCC-14	44.2 (30; 53)	22 (19; 33)
			SAS	99.1 (91; 108)	10 (9; 11)
Hypofract.	23.5	25.9	UT-SCC-14	44.2 (30; 53)	59 (49; 86)
			SAS	99.1 (91; 108)	26 (24; 28)

*Data from [22]. *Abbreviations:* D_{tot} = total absorbed dose of the five fractions, $D_{\text{tot,RBE}}$ = RBE-weighted total dose ($\text{RBE}_{\text{prot}} = 1.1$), $\text{TCD}_{50,\text{phot}}$ = 50 % tumor control dose for photons.

The animals were divided into three dose groups: A control group with no irradiation, a conventional dose group ($D_{\text{prot}} = 1.8 \text{ Gy/fx}$), and a hypo-fractionated group ($D_{\text{prot}} = 4.8 \text{ Gy/fx}$). Table 2.3 gives an overview of the total applied doses, assuming a generic proton RBE of 1.1 for biologically equivalent proton and photon doses [120]. A mixed cohort of SAS and UT-SCC-14 tumors was randomly assigned to each treatment arm (see Figure 2.10). Each arm contained $n = 16$ animals, of which a subset of four animals per arm was used in this work for the vascular architecture characterization. Animals were excluded from further analysis if MRI imaging failed (e.g., due to equipment failure), or if a histologic sample could not be obtained (e.g., if the mouse died during the imaging procedure), or in the case of incorrect irradiation (e.g., if mice removed the hind leg containing the tumor from the fixation clamp).

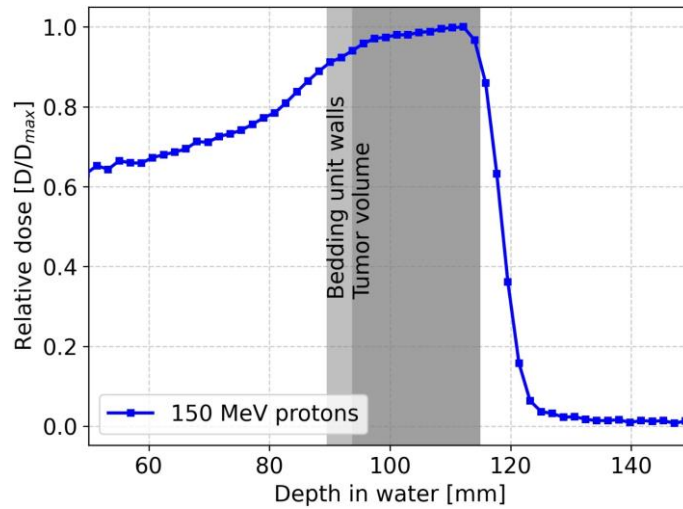


Figure 2.11. Animal irradiation setup. The subcutaneous tumor on the hind leg of the mouse is positioned in a bedding unit and irradiated with a spread-out Bragg peak (SOBP) of 150 MeV protons. The image is reprinted with kind permission of J. Müller (OncoRay, Dresden).

Proton irradiations at a dose rate of 1 Gy/min were performed with an IBA C230 cyclotron at the fixed horizontal beamline of the University Proton Therapy Dresden (UPTD, at the OncoRay, University Hospital Carl Gustav Carus, Dresden). A beam-shaping setup based on the work of [121] and [122] was used for the application of the spread-out Bragg peak depicted in Figure 2.11. The setup was adapted for irradiation of subcutaneous tumors using a 12 mm thick collimator made of aluminum with an aperture of $2.5 \times 5 \text{ cm}^2$, which was placed within proximity to the tumor to avoid irradiation of normal tissue. An in-house-developed (in Dresden) multimodal positioning platform for small animal irradiation was used to fix the hind legs of the mice in the positioning unit with immobilization clamps and to transport the mice to the irradiation site. To avoid painful bruising of the hind legs due to fixation, the mice were carefully examined for restricted mobility after each fraction. The positioning unit was equipped with a red transparent cover for visual control of correct tumor positioning.

Daily dosimetry and *monitor unit* (MU) calibration were performed using a PinPoint 3D ionization chamber type 31022 (PTW Freiburg, Freiburg, Germany) placed in the treatment position in a specially designed surrogate mouse bedding unit. The extent and homogeneity of the field were characterized by irradiating EBT3 films in the same position before animal irradiation.

Sample preparation for histological imaging

The tissue extraction and IHC staining were performed by J. Müller as described in [114, pp. 70–71]. The following description closely follows the referenced work.

Animals were injected intraperitoneally with the hypoxia marker pimonidazole (Natural Pharmacia International, Inc., Research Triangle Park, NC, USA; 0.1 mg/g body weight, dissolved to 10 mg/ml in 0.9 % NaCl) [115]. After 60 min of enrichment time, the perfusion marker Hoechst33342 (Sigma Aldrich, Deisenhofen, Germany; 15 mg/kg body weight, dissolved in 0.05 ml phosphate-buffered saline (PBS)) was injected intravenously [115]. At 60 s after injection of Hoechst33342, the animals were sacrificed by cervical dislocation.

Tumors were excised and cut into two halves. One half was snap-frozen in liquid nitrogen (-80°C) for histological evaluation and the other half was stored in 4 % Paraformaldehyde (PFA) for LSFM analysis. A minimum number of two adjacent $10\text{ }\mu\text{m}$ thick tissue sections were cut from the frozen tumors near the center of each tumor sample. The tissue sections were either thawed and dried at room temperature for 1 h for direct processing or frozen at -80°C for later application.

During the following incubation steps, tissue sections were kept in a dark and humid environment. The dried samples were fixed in acetone at 4°C , dried, and rehydrated with PBS. The sections were then treated overnight at 4°C with a polyclonal antibody against pimonidazole (host species: rabbit, dilution 1:2500, Natural Pharmacia International, Research Triangle Park, NC, USA) and an antibody against mouse CD31 (host species: rat, dilution 1:1500, PharMingen/BD Biosciences, Heidelberg, Germany).

Sections were then washed with PBS and probed with FITC-conjugated polyclonal goat anti-rabbit and TRITC-conjugated polyclonal Goat anti-rat antibodies (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature [115]. The sections were then washed with PBS, incubated with DAKO Fluorescence Mounting Medium (Agilent, Santa Clara, CA, USA), and cover-slipped.

Sample preparation for LSFM imaging

The tissue halves, that were stored in 4 % Paraformaldehyde (PFA) for LSFM analysis as described in the previous section, were used in the following. The *iDISCO+ protocol version from December 2016* [123] was used for sample clearing, i.e. transparentizing the samples while preserving their internal three-dimensional structure [124]. The clearing workflow is described in detail in Appendix B and resulted in a 40 % isotropic volume loss.

The same antibodies against mouse CD31 and pimonidazole as for the histological sections were used for the staining for the UT-SCC-14 and SAS tumors. For the AT1

tumors and the rat brain samples, lectin-FITC (Sigma-Aldrich, St. Louis, MO, USA) was used instead of CD31.

2.3.3. Microscope imaging

Acquisition of histological images

Immuno-fluorescent (IF) images of the tissue sections were obtained using a Zeiss Axio Imager M1, a Zeiss Axio Imager Z2 Fluorescence Microscope, or an Axioscan wide-angle slide scanner at the Light Microscopy Facility of the Center for Molecular and Cellular Bioengineering (CMBC) (all Carl Zeiss AG, Oberkochen, Germany) with a 10× magnification objective, yielding a lateral resolution below 0.44 μm . The sections were scanned with light filters for Hoechst33342, TRITC, and FITC fluorescence signals.

After the IF scans, sections were immersed in PBS to remove the coverslip and subsequent HE staining. Brightfield images of whole sections were then obtained using the microscopes mentioned above or a VENTANA iScan Coreo slide scanner (Roche, Basel Switzerland).

Acquisition of LSFM images

The cleared tumor halves or complete samples (AT1 tumor, rat brain) were glued on a sample holder with Kwik-Sil™ Low Toxicity Silicone Adhesive (VWR, World Precision Instruments). The samples were then scanned using a wet-objective *light-sheet fluorescence microscope* (LSFM) with two excitation lasers perpendicular to the objective, as schematically shown in Figure 2.12.

LSFM offers fast scans of large field of views with deep sample penetration and limited photo-bleaching [123]. On the other hand, increased autofluorescence and limited IHC labeling due to tissue impermeability reduce image quality compared with 2D imaging of histologic sections.

The UltraMicroscope II (LaVision BioTec GmbH, Bielefeld, Germany) with 6 light sheets and infinity corrected optics was utilized for the image acquisition of images with 2x magnification and 2x objective lens (in-plane resolution 2.032 μm) and step size in the transverse plane of 5 μm . Images were acquired as 2x2 or 3x3 tiles in the transverse plane and exported as individual TIF files.

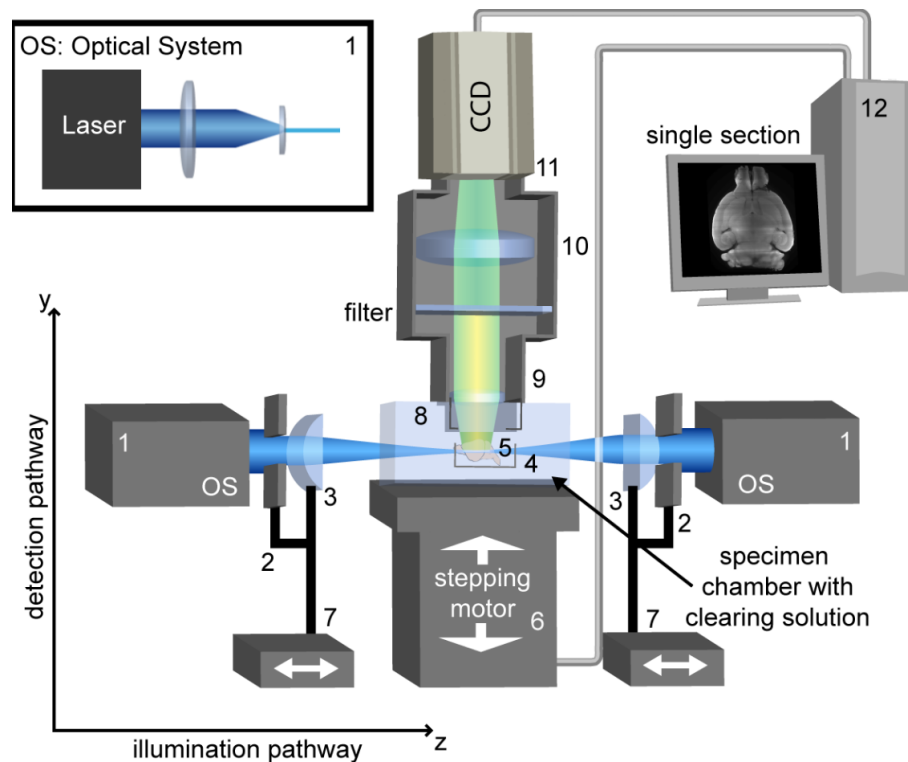


Figure 2.12. Setup of the light sheet fluorescence microscope. The specimen is illuminated from two sides with the optical systems (OS, upper left panel) and consists of a rectangular slit aperture (2) and a cylindrical lens (3). The illumination occurs perpendicular to the detection pathway with a thin sheet of light. Thus, only a slice in the y-direction of the specimen (5), which is fixed with a clamp (6) in the clearing solution, is excited and emits fluorescence. The entire specimen is scanned by moving the 3D-movable stage (6), so that a stack of images can be acquired. The objective (9) projects the emitted fluorescence light through one (or more) tube lens(es) (10) onto a CCD camera (11), while the excitation light is blocked by a matching filter. The acquired single image tile is processed with the microscope software (12). Additional components: (7) Z-stage, (8) Immersion cap.

© 2015 Jährling et al. Image modified from [125], licensed under CC BY 4.0².

2.3.4. Image analysis

2D analysis based on histological images

The 2D image analysis was performed by J. Müller as described in [114, pp. 72–75]. The following description closely follows the referenced work. A semi-automated image-processing workflow was established by J. Müller with the *immuno-fluorescent* (IF) images serving as a basis to measure hypoxia, perfusion, and vasculature parameters. Hematoxylin-and-eosin (HE) stained images were used to distinguish vital from

² <https://creativecommons.org/licenses/by/4.0/>

necrotic regions. Both image types were acquired for the same co-stained IHC slice and then co-aligned using the following workflow for the image processing toolbox *Fiji* [126]–[130], which was implemented as part of the thesis of J. Müller [114]:

The co-alignment using the *elastix* toolbox [131], [132] was performed by reducing the resolution of the image with a higher resolution to match the resolution of the lower-resolution image. Subsequently, the images were thresholded to generate binary masks of the tissue boundaries, cropped and co-aligned using affine transformations (including rotation, translation, and isocenter scaling). The transformation of the masks was then applied to the original images. The transformed images were then used for a pixel classification with the *ilastik* toolkit [133] including background correction, normalization, and segmentation.

Subsequently, the two resulting segmented images were used to calculate the TME parameters. The image, which was based on the HE image, contained the classification of pixels into one of the three labeled categories "background", "viable", and "necrotic". The image derived from the IF image included the four distinct pixel classes "background", "hypoxic" (based on pimonidazole-positive staining), "perfused" (Hoechst33342-positive), "vessel" (CD31-positive).

From these segmented images, the following area-related parameters could be derived for each slice, respectively:

- *Necrotic fraction* (NF): Fraction of necrotic area compared to the total tumor area
- *Hypoxic fraction* (HF): Fraction of pimonidazole-positive hypoxic area compared to the vital tumor area
- *Hypoxic volume* (HV): Product of tumor volume at the time of excision and HF
- *Vascular fraction* (VF, also known as relative vascular area): Fraction of CD31-positive vascular area compared to the total tumor area
- *Perfused vascular fraction* (PVF): Fraction of both CD31-positive and Hoechst33342-positive vascular area compared to the total tumor area

HF, VF, and PVF were manually assessed by four different observers to increase the reliability of the method. In addition to the area-related parameters, the following distance measures were calculated from the combined images:

- *Mean vessel radius* ($\bar{r}$): For each vessel, which is assumed to have an idealized cylindrical shape, an ellipse is fitted to the vessel outline and the length of the ellipses minor axis is considered as the radius.
- *Hypoxic distance* (d_{hyp}): The distance of each pimonidazole-positive hypoxic voxel to the nearest CD31-positive blood vessel.

For each of the two distance measures, the median, mean, and standard deviation were calculated for each histological slice.

3D LSFM image processing for extracting the vascular architecture

The processing of the acquired LSFM images was divided into three steps: Pre-processing, segmentation, and post-processing.

Pre-processing with Fiji and Python

The 3D-scanned fluorescence image stacks were exported with the LaVision Ultramicroscope II software as individual z-slices with 16-bit grayscale information in the *Tag image file* (TIF) format, with z being the direction parallel to the optical axis of the objective. The following steps were performed as a prerequisite for optimal and automated vessel segmentation:

1. Removing all spaces in the filenames using the program *Bulk Rename Utility*³ version 3.3.1.0.
2. Each z-slice consisted of several tiles that were combined to a single image with the function *Stitch Sequence of Grids of Images* [134] of the image processing toolbox *Fiji (ImageJ2)* version 1.53h [126]–[130].
3. The stitched z-slices were combined as a 3D image stack for bulk processing of the entire volume in Fiji.
4. The resulting 3D image was processed in Fiji to enhance the vessel signal:
 - a. The image was convolved with a Gaussian kernel filter of the *Gaussian Blur* (2D) function. A radius of one pixel, i.e., 2 μm for our samples, was chosen as the filter size, where "radius" corresponds to one standard deviation of the Gaussian function, which decays to $\exp(-0.5) \sim 61\%$ of the maximum at this distance [135].
 - b. The non-uniform background of the LSFM image was reduced using the *Subtract Background* function. The background subtraction filter uses a robust histogram-based rolling-ball algorithm [136], described in [137], to correct for nonlinear background, e.g. due to inconstant illumination or artifacts from autofluorescence [138]. The background subtraction algorithm consists of two steps [139]:
 - i. The input image is smoothed with a mean filter (size 3 x 3 pixels) to reduce noise [139]. Afterward, a quadratic sliding window of length L moves through the entire image with a spacing of $L/2$ for each iteration. The sliding window length L should be chosen to be larger than the largest expected object diameter. In our case, the length was therefore set to 25 pixels, i.e., 51 μm for our samples, to avoid filtering out even the largest vessels. For each sliding window, the local histogram

³ <https://www.bulkrenameutility.co.uk/>

of pixel intensities is calculated. Von Neumann boundary conditions are used to compensate for missing pixels for the calculations for edge pixels. The most frequent histogram value for each sliding window is assumed to be the background value for that window. The histogram value is assigned to the central pixel of the sliding window and copied to the newly created background image. For the other pixels for which the background was not explicitly calculated, a bilinear interpolation of the four nearest explicitly calculated pixels is used.

- ii. The background image is filtered with a Gaussian kernel filter with radius $2L$ to remove noise if L is too small or to adjust the background if the concentration of objects is high [138].
5. The image stack was cropped in Fiji based on the bulk tissue boundaries:
 - a. A maximum intensity projection was performed along the z-axis and the resulting image was filtered with the Gaussian *Blur* (2D) function using a radius of 10 pixels, i.e., 20 μm for our samples. The blurring resulted in a slightly overestimated size of the tissue since the tissue edges were smeared. This overestimation of the image edges was in the single-digit pixel range, which was later manually corrected.
 - b. The projection contains one or more filled 2D regions shaped like tissue boundaries. To create a mask of the tissue borders, the regions are binarized with a manually selected threshold for each tissue sample. Thus, the binary mask contains two classes: Tissue areas with pixel values of one and areas outside the tissue or holes in the tissue with values of zero. The manual thresholding is necessary because of the large intensity differences between different samples.
 - c. The command *Fill Holes* was used to close possible holes in the 2D mask. These holes can occur, for example, in tissue volumes with reduced signal, typically found in the center of tumors due to necrosis or decreased diffusion of the fluorescent marker.
 - d. If the mask consists of more than one non-contiguous region, e.g., due to noise or due to some loose tissue pieces outside the bulk volume, all but the largest region were removed using the *Analyze Particles* function.
 - e. The remaining single 2D region of the mask is then used to uniformly crop the tissue edges in the original 3D image for each individual z-slice. For this purpose, the image size is reduced to the shape of the mask (described by a polygon) and the voxels outside the region are set to zero.
6. The intensities of all slices were individually normalized using the *Enhance contrast* function [140] to improve signal homogeneity for segmentation. To this end, the *normalize* option was enabled, which, in the case of our 16-bit images, converts the pixel values of the image to the range [0, 65 535].

7. The 3D image stack was then converted from the TIF format to chunked *hierarchical data format 5* (HDF5®) [141] with the image axis order TZCYXS using the libraries *tifffile 2020.10.1*⁴ and *h5py 2.10.0*⁵ [142] of the *Python 3.8.5* programming language [143]. The HDF5 allows the *ilastik* toolkit [133] to effectively process a large image on a chunk-by-chunk basis.

Segmentation with *ilastik*

For vessel segmentation, the *Pixel Classification* workflow of the interactive learning and segmentation toolkit *ilastik 1.3.3post3* was used. Training and segmentation were performed separately for each sample, as the intensity and noise level varied widely between different images.

1. In the *Feature Selection step*, useful features such as color/intensity, edge filters, and texture descriptors were selected. The selected features can be applied in 2D or 3D on different scales. The scales correspond to those of the respective smoothed version of the base image using multiple Gaussian kernel filters with user-defined standard deviations. In the present work, the parameter combinations listed in Figure 2.13 were used.

	• σ_0	• σ_1	• σ_2	• σ_3	• σ_4	• σ_5	● σ_6
Compute in 2D/3D	3D	3D	3D	3D	2D	2D	2D
Sigma	0.30	0.70	1.00	1.60	3.50	5.00	10.00
► Color/Intensity	☒	☒	☒	☒	☒	☒	☒
► Edge	☐	☒	☐	☒	☐	☐	☐
► Texture	☐	☒	☐	☒	☐	☐	☐

Figure 2.13. Feature combinations for the *ilastik* image segmentation. Sigma = standard deviation of the Gaussian filter.

2. In the interactive *Training step* for the classifier, two different labels (vessels, and background) were defined and annotated in the images, which takes approximately [30, 60] minutes per tissue sample.
3. In the *Prediction Export step*, a *random forest classifier* trained on the user annotations was used to segment the entire sample. The resulting probability map was exported as an unsigned 8-bit image in the HDF5 with renormalization of the voxel values from [1, 2] to [0, 255]. Thus, the pixel intensity values from 0 to 255 represent the probability from 0 to 100 % that a voxel is a vascular voxel, or with 100 % minus this probability, that it is a background voxel. Segmentation and export together took about 12 min/GB on a personal computer equipped

⁴ <https://pypi.org/project/tifffile/>

⁵ <https://www.h5py.org/>

with an Intel® Xeon® CPU E5 quad-core processor with 3.50 GHz and 64 GB RAM.

Post-processing with Fiji and MATLAB

1. The 8-bit 3D image was converted from HDF5 to TIF format using the Python libraries *tiffio* and *h5py* and the image axis order TZCYXS.
2. The resulting 8-Bit TIF image was thresholded with a cutoff value of 128 to generate a binary vessel image.
3. The Fiji function *3D Fill Holes* was applied to fill cavities inside the vessels that can be generated by the segmentation.
4. The following three Fiji functions were applied to connect interrupted vessel segments:
 - a. *Dilate (3D)* with a dilation radius of one voxel to slightly increase the vessel extension in the whole image.
 - b. *Median 3D* filtering with a radius of two pixels in x- and y-direction and one pixel in the z-direction to fill gaps between vessel segments. The median filter was chosen to preserve the original vessel boundaries.
 - c. *Erode (3D)* using a voxel distance of one voxel to reduce the vessel extension to its original size before the dilation in a) while preserving the new connections between vessels through the median filtering in b).
5. Step 5 of the pre-processing pipeline was repeated here for the 8-bit vessel image to mask the tissue borders by setting the voxels outside the borders to a value of “100” to distinguish them from background (“0”) and vessels (“255”).
6. The tissue boundaries, thus globally defined for all z-slices, were not representative for all z-slices due to the irregular tissue shape and were therefore refined using a self-written script in the *ImageJ Macro 1.x* language: The unmarked outer areas were manually filled in each z-layer using a spacing of 40 z-slices (i.e., 200 μm) in Fiji. Besides that, inner tissue areas with very weak or no signal, e.g., due to necrosis or insufficient diffusion of the antibody, were also masked.

Empirical calculations using the 3D LSMF vascular architecture

To assess unique features of a network, such as the vascular network, in this case, a range of metrics should be used [144]. Thus, after capillary grid extraction, described in the next section, three types of capillary parameters were analyzed: (i) vascular parameters that are comparable with histological (2D) parameters, (ii) conventional 3D vascular parameters, and (iii) network properties using a graph-theoretic approach.

Extracting the capillary grid from partial volumes

1. The image stack was partitioned in *MATLAB Version 9.8* [101] into cubic subvolumes of 0.512 mm^3 each, i.e. with a side length of $800 \mu\text{m}$. This allows for faster image analysis in parallel mode and the selected image size already has the optimal dimensions for the oxygen diffusion simulations with the TOM. Three different types of images were generated for each subvolume. These images listed below were then used for different evaluations:
 - a. The uint32-bit *object map* contains all segmented vessels. All voxels belonging to a particular vessel are labeled with an integer number unique for each vessel.
 - b. The binary *skeleton* contains the center lines of the vessels.
 - c. The binary *capillary grid* that is generated by interpolating all capillaries of the object map to an isotropic voxel size of $125 \mu\text{m}^3$ (side length $5 \mu\text{m}$) and is used as input volume for the TOM.
2. The skeleton images are evaluated with the *Analyze Skeleton (2D/3D)* function [145] in Fiji, with the option *pruning of the shortest branch* enabled. With this, parameters such as the average branch length, etc. are determined and exported as a CSV file.

Calculation of parameters comparable with histology

The *radii of the vessels* were determined from the object map in Fiji as follows: The *3D Distance Map* function of *3D ImageJ Suite* [146] was applied to the object map with the option "map = EDT" (EDT = Euclidean Distance Transform) to determine the shortest Euclidean distance from each voxel to the nearest background voxel. Then the distance map image was multiplied by the matching skeleton image to obtain only the distances of the voxels of the vessel center to the background. These voxels provided a good approximation for the vessel radius and were exported as a *nearly raw raster data* (NRRD) image file. Thus, all values for the radius along the centerlines of all capillaries in a subvolume were available and were averaged for each vessel for later analysis.

The *vascular fraction* (VF) is determined for each subvolume from the object map as the number of capillary voxels (labeled with values larger than zero) divided by the total number of voxels.

Calculation of the conventional 3D vascular parameters

Each vessel is defined as the totality of all interconnected vascular voxels. Here, interconnected means that the voxels are 26-connected in 3D, i.e., all directly adjacent voxels in a voxel distance of less or equal $\sqrt{3}$ are combined. The MATLAB function

bwlabeln [147] was used to discriminate the different vessels. Each segment of a vessel is called a *branch*, which is connected to other branches at intersection points, so-called *nodes*. Thus, each node is either a *branching point*, where at least three branches meet, or an *end point* of a branch that is not connected to other branches. The number of branches starting from a node is a natural number n in the range $\{1, 3, 4, 5, 6, \dots, n\}$, and is called *node degree*.

If a branch makes an abrupt change in direction, nodes may be misclassified during branch detection with the *Analyze Skeleton (2D/3D)* function [145] of Fiji. In this case, this change in direction is counted as a node of degree two, although it presents only a single branch. This artificially shortens some of the measured branch lengths and increases the total number of nodes. To maintain comparability of our results with those of Hahn et al. (2019) [134], this issue has not been corrected.

The conventional parameters can be grouped in density, branching, and distance measures, as listed below.

Density parameters

- *Density of branches* (ρ_B): Quotient of the number of branches, i.e., vessel segments, and the volume
- *Total vessel density* (ρ_V): Quotient of the number of vessels, i.e., isolated objects, and the volume
- *Vessel length density* (ρ_{VL}): Quotient of the total length of all vessels and the volume

Branching parameters

- *Mean branch length per vessel* ($\bar{l}_{B,V}$): Lengths of all branches of a vessel averaged for that vessel
- *Tortuosity* (τ): Curvature of a branch, i.e., its length divided by the Euclidean distance of its two endpoints
- *number of branches per vessel* ($N_{B,V}$): Number of branches of one connected vessel object
- *number of branches per node* ($N_{B,N}$): Number of branches that join at a connecting voxel

Distance measures

- *Tissue-to-vessel distance* (TVD): Euclidean distance of a tissue voxel to the nearest vessel center line voxel, i.e., a voxel of the vessel skeleton
- *Inter-vessel distance* (IVD): Shortest Euclidean distance from the center line of one vessel to the center line of the nearest adjacent vessel

Graph-theoretical network parameters

The vascular architecture determined from the LSM images can be considered as an undirected network (no specific direction of “information” flow), so that analysis methods from graph theory can be employed to compare the individual subvolumes. A *graph* in this case refers to a vessel with all its connected branches. The individual branch points and ends are called *nodes* of the network. The misclassification of nodes with degree two (previously mentioned in Section 2.3.4) also impacts the accuracy of the network features. However, this problem is only relevant for a small number of nodes, since on average only (2.3 ± 1.7) % of all branches of all subvolumes exhibited grade two. The connections of the individual nodes, i.e., the branches, are called *edges* in graph theory, which may be weighted using their respective branch length.

The term *unweighted* for graphs denotes that all edges have the same length of one unit distance. Thus, the information about edge lengths (i.e., branch lengths) is not used and the unitless distances calculated for unweighted graphs are independent of the branch lengths. In contrast, *weighted graphs* additionally include the branch lengths as weights for the edges, so that distance calculations are dependent on the branch lengths.

For the experimental architectures used in this work, the vessel architecture is converted into network graphs with the MATLAB function *graph* that generates the graphs from the list of branch endpoints from the *Analyze Skeleton (2D/3D)* function (Fiji). Four groups of network parameters were then analyzed (node parameters, graph size parameters, network connectivity parameters, and branching properties of the network), as introduced in the following.

Node parameters

- *Density of branching nodes* (ρ_B): Quotient of number of nodes (with a node degree of at least three) and volume
- *Density of end nodes* (ρ_E): Quotient of number of nodes (with a node degree of one) and volume
- *Fraction of end nodes* (f_E): Ratio of number of end nodes to the total number of nodes
- *Node degree* (k): equivalent to *the number of branches per node* (see previous section “Calculation of the conventional 3D vascular parameters”)

Graph size parameters

- *Graph density* (ρ_G): Quotient of the total number of graphs (each based on an isolated vessel) and volume
- *Density of large graphs* (ρ_{LG}): “large graphs” refers to the subset of graphs that contain at least 10 edges, i.e., connected branches. The value of 10 is an arbitrarily chosen threshold to exclude short graphs from the analysis, such as interrupted graphs at the volume borders or incorrectly labeled vessels.
- *Fraction of small graphs* (f_{SG}): Quotient of the number of graphs smaller than 10 edges and the total number of graphs
- *Characteristic path length* (L): Mean of the shortest path lengths $d_{i,j}$ between all node pairs i and j of a graph with n nodes:

$$L(G) = \frac{1}{n(n-1)} \sum_{i,j=1; i \neq j}^n d_{i,j}. \quad (2.43)$$

The shortest path length $d_{i,j}$ describes the shortest number of (weighted) edges between node i and node j .

- *Network Radius* (R): minimum of the shortest path lengths between all node pairs in a graph
- *Network diameter* (D): maximum of the shortest path lengths between all node pairs in a graph

Network connectivity parameters

- *Clustering coefficient* (C) of a node: Quotient of the number of direct connections between direct neighbors of the node and the maximum possible number of connections. C is therefore normalized between zero and one.
- *Average efficiency* (E): Distance measure for a graph G , that is calculated with the inverse of the shortest path length $d_{i,j}$:

$$E(G) = \frac{1}{n(n-1)} \sum_{i,j=1; i \neq j}^n \frac{1}{d_{i,j}} \quad (2.44)$$

- *Local efficiency* (E_{loc}): Distance measure, which uses the local subgraph G_i (direct neighbors of node i excluding node i itself) for the efficiency calculation:

$$E_{loc}(G) = \frac{1}{n} \sum_{i \in G} E(G_i) \quad (2.45)$$

Thus, the local efficiency is an alternative to the clustering coefficient and characterizes how well information is exchanged by its neighbors when a node is removed from the network.

- Global efficiency (E_{glob}): Quotient of the average efficiency of a selected graph and the average efficiency of an ideal graph having all possible edges (i.e., all its nodes are directly connected):

$$E_{\text{glob}}(G) = \frac{E(G)}{E(G^{\text{ideal}})} \quad (2.46)$$

Branching properties of the vascular network nodes

Random graph networks typically exhibit an exponential decay of the frequencies of the node degrees [148], [149]. To artificially generate these random graphs, the *Erdős-Rényi model* [150], [151] is usually used. However, in *real-world networks*, such as the vascular network studied here, a power-law decay is observed, since a small number of highly connected nodes, i.e., so-called *hubs*, leads to a long tail of the node degree frequency distribution.

Networks that exhibit this power-law behavior can be classified as *scale-free networks* [152]. Scale-free means that the power-law function maintains the same functional form for all scales, i.e., that only a multiplicative factor changes [153].

The term *ultrasmall* is also used for scale-free networks due to their very short connections between individual nodes [154]. Thereby, the characteristic path lengths are reduced compared to other classes of networks. Analytical studies by [154], [155] have shown that the reduced characteristic path lengths lead to the following dependence of the characteristic path length L on the number of nodes N :

$$L = \log \log N. \quad (2.47)$$

Consequently, hubs are of central importance in these types of networks and a functional failure of a hub in real tissue can have far-reaching effects.

2.3.5. TOM simulations based on the 3D vascular architecture

Section 2.2.4 described the extraction of the vascular architecture of the different tissue samples. The vascular architectures were used as an input for the TOM simulations to compute the oxygen distributions of the experimental samples.

3D experimental simulation grids

For each sample, the object maps, i.e., the volumetric subvolumes with labeled vessels as described in Section 2.3.4, were used as the basis for extracting the vessel network. The object maps contained the binary vessel information (“1” for vessel voxel and “0” for background) and had already been interpolated to an isotropic voxel size of 5 μm in a previous step. Thus, the object maps with a cubic volume of $(0.8 \text{ mm})^3$ could be directly imported into the TOM and used as simulation grids. The TOM simulations

were then performed using the default parameters of the TOM version 2021, as listed in Table 3.5. of Section 3.1.5.

2D synthetic simulation grids

For a comparison of the complex experimental vascular networks to the simplified linear capillaries of the TOM 2013 version, as used in [27], the 2D capillary grids were generated based on the data of the 3D subvolumes:

- The vascular fraction was computed from the object map and used as an input parameter.
- A vessel radius of five micrometers was chosen for all samples since it matched well the experimental results in Table 3.8, which showed similar mean radii for all samples that were distributed around this value. This was a distinctive change compared to the standard settings of the TOM 2013 version (Table 2.1), which used a radius of 10 μm based on data on colon adenocarcinoma from [37].
- For each matching 3D volume, 20 independent 2D simulations were performed on a square simulation grid with an 800 μm side length.

The simulations on the 3D experimental and 2D synthetic simulation grids were performed in parallel using the MATLAB Parallel Computing Toolbox [156]. Each of the 1750 simulations for the experimental volumes lasted 1:54 min and a total volume of 0.9 cm^3 was simulated in 2 days and 8.5 h. The associated 35 000 simulations on the synthetic grids took one second per simulation and thus a total area of 224 cm^2 was simulated in 1 h and 20 min.

3. Results

Three major questions were addressed in the present work:

- (i) Are the numerical solutions of the DRE with the TOM numerically accurate? Which settings reveal optimal results in terms of accuracy and calculation efficiency?
- (ii) Which parameters characterize the vascular architecture of the examined human HNSCC xenografts and their development under irradiation?
- (iii) What is the impact of the experimental 3D vessel architecture on tumor oxygenation? Is the simplified parallel line architecture of TOM sufficient to model these 3D differences in a 2D setting?

3.1. Numerical accuracy of the diffusion-reaction equation solutions

The numerical validation is divided into three steps. First, the oxygen distributions generated by the TOM are compared with published results. Then an analytical solution of the diffusion-reaction algorithm is introduced. Based on this analytical solution, the TOM is numerically optimized.

3.1.1. Comparison of TOM histograms with other studies

The studies of Warren and Partridge in 2016 [94] and Paredes-Cisneros in 2018 [157] verified the results of Espinoza et al., 2013 [27], that a simple 2D parallel linear capillary architecture leads to a comparable oxygen distribution as a 3D parallel and orthogonal architecture. Furthermore, both authors verified that 3D capillaries with isotropic orientation can be replaced by parallel 2D distributions as well. But although the oxygen histograms in all studies were based on 3D parallel linear capillaries with comparable biological input parameters, the resulting oxygen histograms in Figure 3.1 show some major deviations.

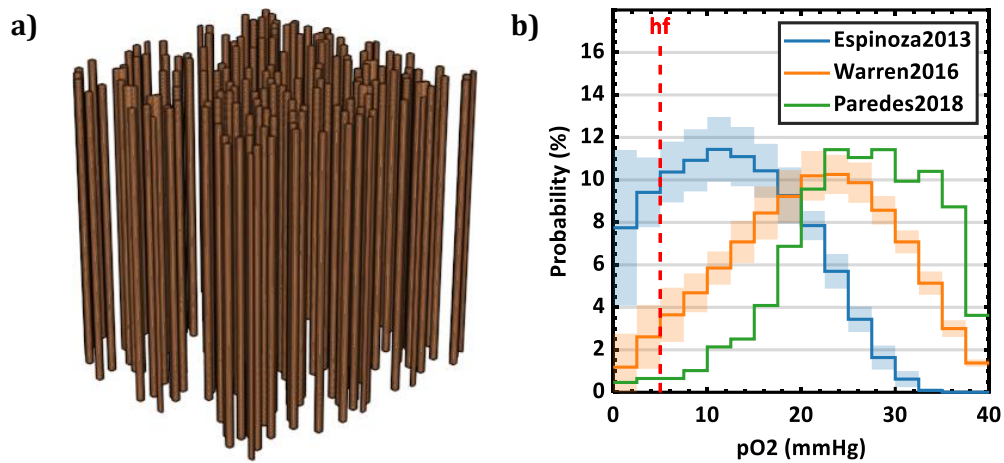


Figure 3.1. a) A cubic simulation volume of 1 mm^3 with parallel linear capillaries and a VF of 5 % was used for the oxygen distribution simulations. b). The oxygen distributions show the means $\pm$ standard deviations of 20 independent simulations. In the case of Paredes2018, a reduced number of 10 simulations was used and no data for uncertainties were available. The numerical parameters for the individual curves are listed in Table 3.1. and the data were obtained from the cited publications except for Espinoza2013, where the data was generated as part of this work.

A reason for the deviations is that the authors used different numerical simulation settings, as listed in Table 3.1. The two main methodological distinctions are the voxel size (i.e., the mesh width) and the numerical method to solve the DRE. Thus, Figure 3.1 b) shows that the oxygen distributions are shifted to higher oxygen levels with an increasing grid resolution from 20 [27] to 10 [94] and $5 \mu\text{m}$ [157]. This is an indication of a possible dependence of the oxygenation on the voxel size.

Table 3.1. Simulation settings for Figure 3.1 b).

Parameter*	Value (%)		
	Espinoza2013 [27]	Warren2016 [94]	Paredes2018 [157]
Numerical scheme	Particle strength exchange	Finite differences	Finite elements
Boundary condition at the simulation volume end	Periodic	Periodic	Von Neumann*
Voxel size	$20 \mu\text{m}$	$10 \mu\text{m}$	$5 \mu\text{m}$
Grid volume	1 mm^3	1 mm^3	$(0.5 \text{ mm})^3$
Number of independent simulations**	20	20	10

*No oxygen transport through the simulation volume boundary.

**Each simulation volume is based on an independent simulation volume with randomly placed capillaries.

The mismatch of the histograms has a biological relevance, which can be seen in Figure 3.2 that shows simulated survival curves based on the histograms in Figure 3.1 b).

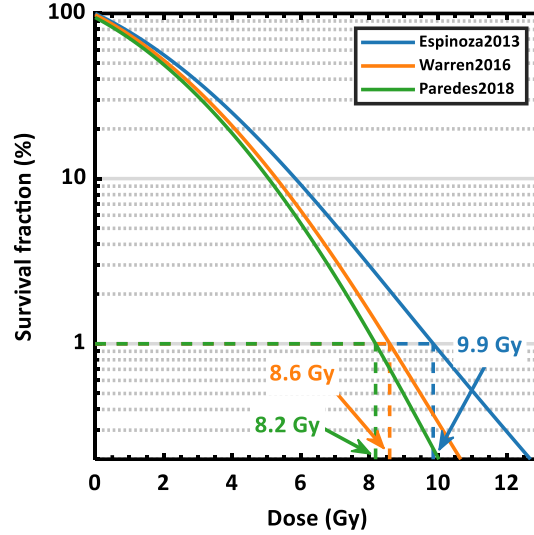


Figure 3.2. Survival curves of the same synthetic tumor, based on the oxygen histograms in Figure 3.1. The dose enhancement ratio (DER) is marked as an iso-effective line at a 1 % survival fraction meaning that different doses lead to the same biological endpoint (fraction of surviving cells). The curves were calculated based on the OER-enhanced LQM formula (2.17) with the radiation sensitivity parameters $\alpha = 0.273 \text{ Gy}^{-1}$ and $\beta = 0.045 \text{ Gy}^{-2}$ of squamous cell carcinoma data from [158], yielding $\alpha/\beta = 6.07 \text{ Gy}$. The OER was calculated with Equation (2.16) using the parameters $k = 3 \text{ mmHg}$ and $m = 3$ from [159].

A quantitative comparison of the histogram and survival curve differences was summarized in Table 3.2, including the dose enhancement ratio (DER, introduced in Section 2.2.4) as a meaningful criterion for estimating the biological relevance. The DER indicates that a tumor simulated with the oxygen histogram from the Espinoza2013 code would require a 15 % higher dose to achieve a 1 % survival compared to the Warren2016 histogram or 21 % more compared to the Paredes2018 histogram. The difference between the Warren2016 and Paredes2018 histograms is less pronounced with a 5 % higher dose but would still result in a higher TCD_{50} for the Paredes2018 simulation. To understand the mismatch of the histograms and to evaluate the impact of voxel size and other parameters, they were further investigated in the following.

Table 3.2. Quantitative comparison of the biological relevance of the histogram differences in Figure 3.1. All parameters are calculated using the frequency bins of the histograms, except the DER that is based on the survival curves in Figure 3.2.

Parameter*	Value		
	Espinoza2013 vs. Warren2016	Espinoza2013 vs. Paredes2018	Warren2016 vs. Paredes2018
MAD (%)	4.6	7.4	2.9
MaxAD (%)	7.0	10.3	5.7
EMD (%)	22	35	14
DER for 1 % SF	1.15	1.21	1.05

* For a description of the parameters, see Section 2.2.4.

3.1.2. Comparison with an analytical solution of the diffusion-reaction equation

None of the authors in Figure 3.1 reported if they evaluated the numerical accuracy of their solutions of the diffusion-reaction equation (DER, Equation (2.24)). To understand the differences of their oxygen histograms in Figure 3.1 b), the numerical results of the TOM were first validated against an analytical solution of the DRE. Based on this comparison, the numerical parameters of the PSEM and FDM algorithms were optimized to achieve numerically accurate results.

Generally, when deriving an exact analytical solution of the DRE as in [74], [80], [84], it is assumed that the cellular oxygen consumption is independent of the oxygen availability. However, the assumption of an oxygen consumption dependent on the oxygen availability according to the Michaelis-Menten relationship in Figure 2.6 is a more realistic assumption than constant oxygen consumption. Solving the DRE with an availability-dependent consumption is thus the preferred method for both validating the TOM and optimizing the numerical algorithm parameters. In the present work, therefore, the solution of Grimes et al [84] was used, who, to our knowledge, are the only ones to have published an analytical solution for realistic oxygen consumption kinetics.

Analytical solution for the DRE of Grimes et al.

Grimes et al. [84] used a single cylindrical capillary, as shown in Figure 3.3, in a cylindrical coordinate system to simulate oxygen flow from a capillary into tissue. A simplification in this approach is that the direction of oxygen flow is always perpendicular to the capillary wall. This simplified model is known as the widely used *Krogh-Erlang model* [59], [80], [160], established as a theoretical concept by August Krogh in 1919 [161]. The complete derivation of the analytical solution described in [84] can

be found in Section A of the Appendix. Grimes et al. did not provide the analytical solution in the publication because “the expression was long and unwieldy” [84]. However, Grimes kindly provided his code in the Mathematica format [162] to recalculate his solution for this work.

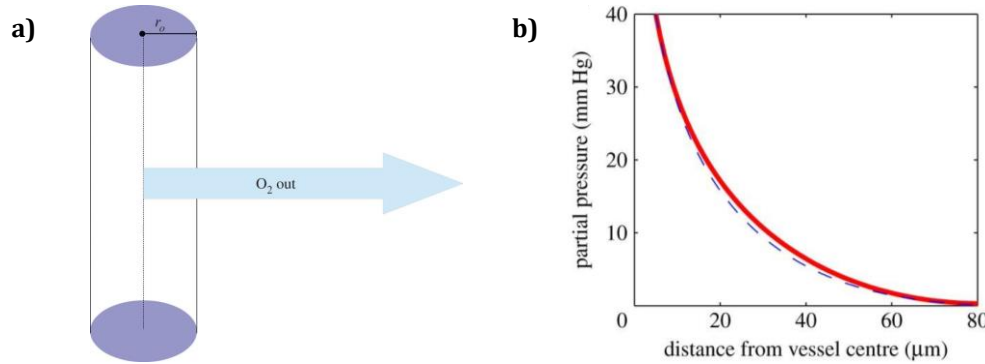


Figure 3.3. Analytical solution of the diffusion-reaction equation. a) Geometry of a Krogh-type capillary with oxygen diffusion perpendicular to the capillary wall. b) Calculated diffusion profile of a capillary with a radius of 5 μm oxygenating the tissue up to about 80 μm assuming a Michaelis-Menten consumption (Equation (2.27)). The figure indicates a good agreement between the approximated solution (red) and a numerical solution (blue). Images from [84], licensed under CC BY 4.0⁶.

Comparison of the diffusion profiles of TOM and analytical solution

Table 3.3. Settings for the TOM simulations. Parameters were chosen to match those of the analytical solution of [84]. In contrast to [84] who used a cylindrical grid, however, a Cartesian grid was used here.

Parameter	Value
Boundary condition	Dirichlet
p_{O_2} at simulation volume boundary	0 mmHg
p_{O_2} at capillary wall	40 mmHg
Grid size*	0.4 x 0.4 mm ²
Capillary radius**	0 μm

* Using a 2D grid results in diffusion only perpendicular to the capillary and thus complies with the respective condition in the Krogh model.

** The TOM 2013 version uses a point source as a capillary. Grimes et al., on the other hand, modeled a capillary with a spatial expansion with a radius of 10 μm . Their solution was thus shifted to the coordinate origin to be comparable with the TOM solution.

In this thesis, the simulation conditions for the analytical solution were adapted to the same set of parameters as in the standard simulations of the TOM 2013 version [27],

⁶ <https://creativecommons.org/licenses/by/4.0/>

using the Mathematica code of Grimes et al. The simulation parameters in Table 3.3 were then used for a TOM simulation with the same capillary geometry as the analytical solution of [84].

The original TOM 2013 version (PSEM) and the updated TOM 2015 version (FDM), introduced in Section 2.2.3, were both compared with the analytical solution of Grimes et al. [84] in Figure 3.4. The TOM 2013 (blue curve) shows the smallest deviations from the analytical solution ($MAD = 2 \text{ mmHg}$, $MaxAD = 6.0 \text{ mmHg}$) compared to the 2015 version in green (2.7 mmHg and 7.2 mmHg) and the TOM 2013 version with a reduced voxel size in orange (3.7 mmHg and 9.7 mmHg).

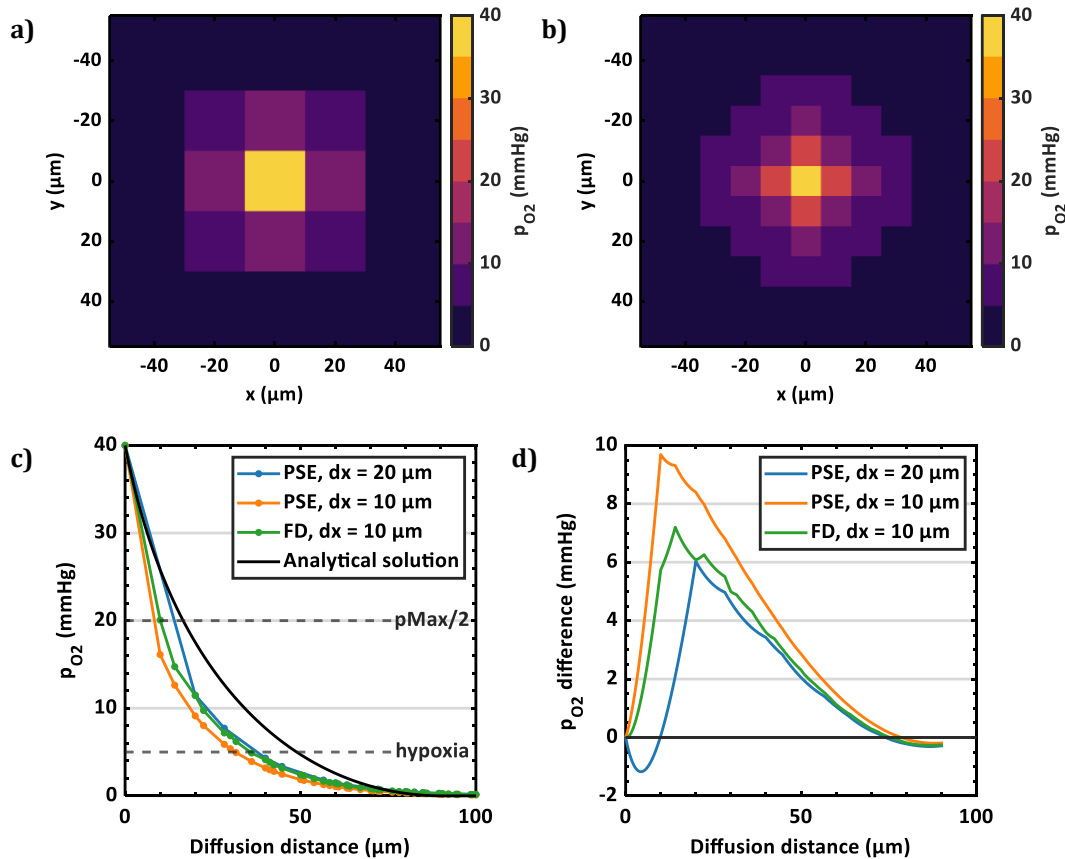


Figure 3.4. Radial oxygen diffusion profile of a single capillary modeled as a point source. a) TOM 2013 version with PSEM and $20 \mu\text{m}$ voxel size. b) Updated TOM 2015 version with FDM and $10 \mu\text{m}$ voxel size. c) Diffusion profile perpendicular to the capillary axis starting at the capillary center. Blue: TOM 2013 from a), Orange: TOM 2013 version with the same voxel size and termination condition as TOM 2015, Green: TOM 2015 from b) d) Interpolated deviation from the analytical solution of c) that are the smallest for the blue curve.

This test was repeated for various voxel sizes, however, the deviations from the analytical solution did not decrease further (data not shown). This means that other factors affect the accuracy of the simulation, such as the finite diameter of the capillary

and the shape of the capillary cross-section, which were examined in the next sections.

3.1.3. Improvements of the TOM accuracy for a single capillary

Influence of the finite capillary volume on the oxygen distribution

So far, the oxygen distributions were simulated in the TOM assuming that the capillaries are linear objects with a point-like cross-section (see Figure 3.5). Thus, their spatial extent on the simulation volume is neglected as well as their cross-sectional shapes.

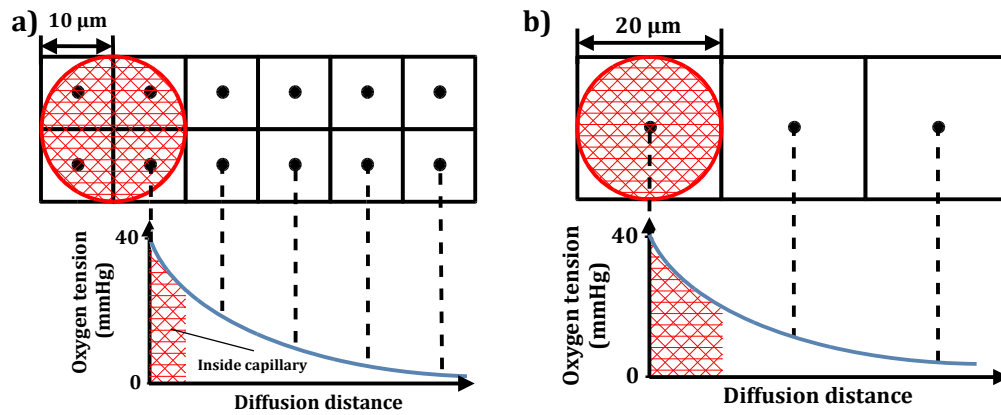


Figure 3.5. Cross-section of a circular capillary shown superimposed over two simplified simulation grids with voxel sizes of a) 10 μm and b) 20 μm . The grid pixels are shown as squares with the grid points in the center. The shaded areas illustrate the extent of an ideal circular capillary with a diameter of 20 μm . The corresponding diffusion profiles show interpolated curves between the highlighted grid points. The circular capillary fills only 79 % of the area of a square with the same length as the capillary, as shown by the “capillary pixel” in panel b). Diffusion starts in the center of the pixel in panel b), while the better approximation of the circular capillary shape in panel a) results in diffusion starting closer to the capillary edge. Since a constant oxygen tension of 40 mmHg is assumed in the TOM simulations, case a) is more consistent with this assumption.

In the following, different capillary cross-sections were investigated to achieve the best agreement with the analytical solution of [84]. For the point-like capillary cross-section, the same simulation parameters as in the previous section (Table 3.3) were used. For the square form a side length of 20 μm was used, the same value as the diameter of the capillary in the analytical solution. Thus, all voxels with a distance between their centers less than or equal to the side length were classified as belonging to the capillary. This means for example that a capillary of a grid with a voxel size of 20 μm or 10 μm is represented by 4 or 16 voxels, respectively.

As shown in Figure 3.6, the capillaries with a square cross-section already agree quite well with the analytical solution. This could be because the p_{O_2} concentration is now assumed to be 40 mmHg over the entire capillary cross-section and not only in the center, resulting in a better space-filling of the capillary. Nevertheless, the maximum deviations of up to 7.5 mmHg, i.e., 19 % of the maximum oxygen tension, are still quite large, indicating that there are further important factors.

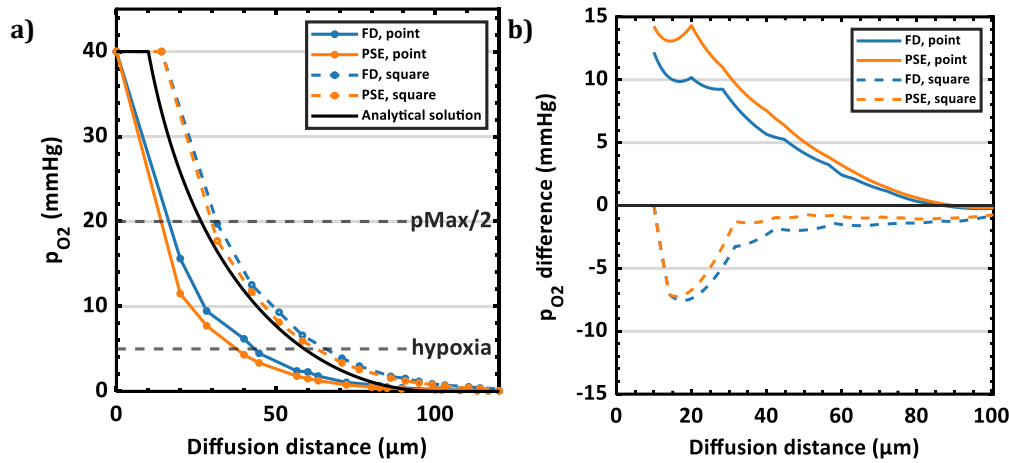


Figure 3.6. Impact of different cross-sectional shapes for a single capillary. a) Radial diffusion profiles of the point and square shapes for both numerical methods implemented in TOM as well as for the analytical solution. A maximum p_{O_2} of 40 mmHg was assumed for the entire cross-section of the square capillary, and the point-formed capillary only at zero distance. b) The difference of the simulated from the analytical solution from panel a) is similar for both numerical methods. The square capillaries overestimate the p_{O_2} (MAD = 2.7 mmHg for the FDM and 1.9 mmHg for the PSEM), while the point sources lead to an underestimation (4.2 mmHg and 5.4 mmHg).

Additional influence of circular vs. squared capillary shapes

If the capillary volume is considered and a capillary with a diameter of 20 μm is to be represented with a voxel size of 20 μm , then this is only possible with a square cross-sectional shape. Only at smaller voxel sizes, it is possible to approximate a circular shape that would more closely resemble natural morphology.

Both shapes were consequently evaluated for a single capillary using different smaller voxel sizes in Figure 3.7. The figure illustrates that the profiles for circular and square cross-sections agree better with the analytical solution than those of a point shape. For voxel sizes of 6 μm and below, no further change in maximum diffusion distance is seen, and the values for circular and square shapes are comparable with 105 μm and 110 μm respectively. The resulting mean deviation from the analytical solution is less than 1.5 mmHg (3.7 % of the maximum p_{O_2}). In contrast, point-shaped capillaries show a mean absolute error almost twice as large and show a clear voxel size dependence of the maximum diffusion distance and the mean absolute error.

Despite the better agreement for smaller voxel sizes, the numerical solution does not fully converge to the analytical solution with a further reduction of the voxel sizes. Thus, there seems to be an additional factor affecting the accuracy of the numerical solutions. In addition to the numerical effects discussed above, the voxel size can also affect (i) the stability and (ii) the termination condition of the simulation when using the TOM with multiple capillaries. These two aspects were further analyzed below and the TOM implementation was updated to be independent of the voxel size.

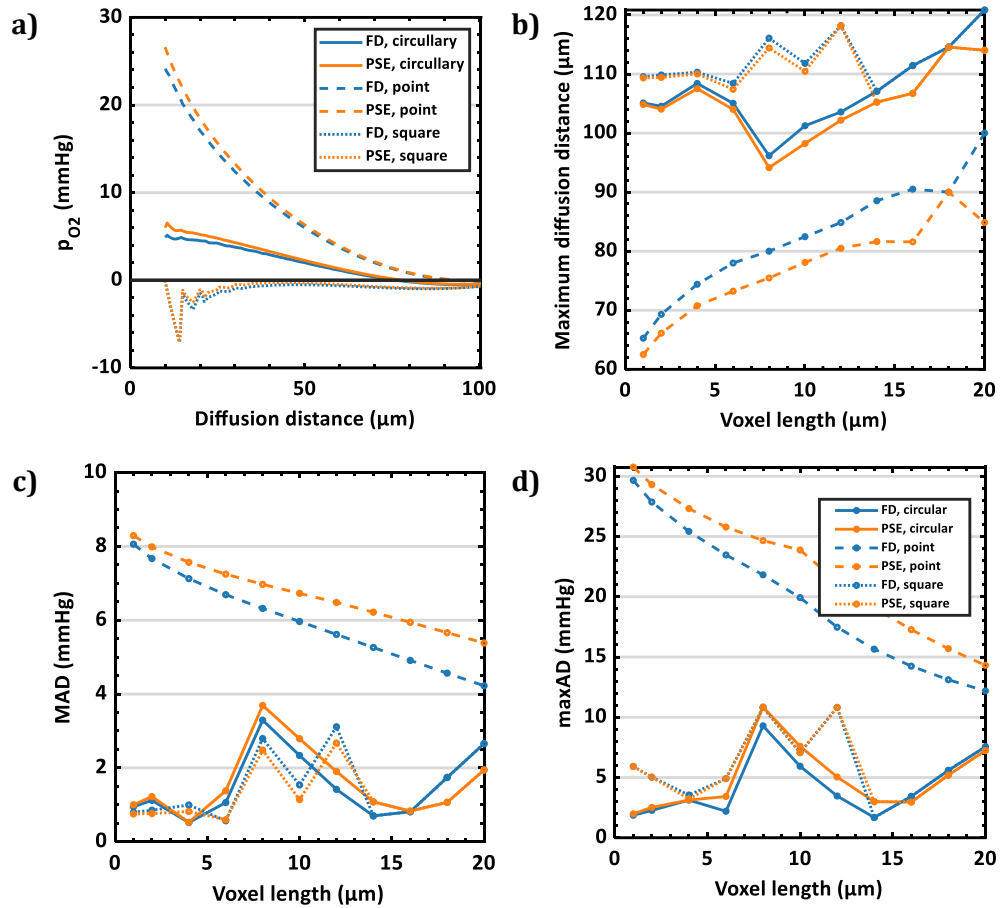


Figure 3.7. Dependence of the deviation of simulated from analytical radial diffusion oxygen profiles on different parameters for different capillary geometries and voxel sizes. a) The difference of the radial profiles from the analytical solution for the example voxel size of $5 \mu\text{m}$. A large underestimation is visible for point-shaped capillaries, while circular and square and capillaries show a much smaller under- and overestimation, respectively. b) The maximum diffusion distance was defined as the distance at which the p_{O_2} drops to 1 % of the initial capillary p_{O_2} , i.e., 0.4 mmHg . This arbitrary limit was necessary since the numerical solution, unlike the analytical solution does not drop exactly to zero. Panels c) and d) show the mean and maximum absolute deviations from the analytical solution vary more for circular and square capillaries at voxel sizes larger than $6 \mu\text{m}$. However, they are smaller by more than a factor of 1.5 than for point-like capillaries.

Courant-Friedrichs-Lewy Condition

The *Courant-Friedrichs-Lewy* (CFL) condition [163] gives a criterion for a stable relationship between the spatial increment dx and the time steps dt for a numerical solution of a partial differential equation. For both methods used in this work (PSEM and FDM), this criterion must be fulfilled to ensure numerical stability and accuracy. The *CFL condition for the one-dimensional case* is defined as

$$C = u \frac{dt}{dx} \leq C_{max} \quad (3.1)$$

with the Courant number C and the velocity u . For a given voxel size/mesh width dx the CFL condition sets a limit to the distance $u \cdot dt$ that the solution can travel in a single time step. Meeting this condition prevents a loss of physical information that would occur otherwise. The threshold C_{max} depends on the numerical solving scheme and typically must be smaller than one for explicit schemes.

For diffusion problems, the velocity u in Equation (3.1) can be replaced by $2D/dx$ using the *Einstein-Smoluchowski Equation* (2.21), which leads to

$$C = 2D \frac{dt}{dx^2} \quad (3.2)$$

for the one-dimensional case and

$$C = 2D \sum_i^n \frac{dt}{dx_i^2} \quad (3.3)$$

for the n -dimensional case. This can be simplified for a spatially isotropic mesh in three dimensions to

$$C = 6D \frac{dt}{dx^2}. \quad (3.4)$$

To define the time steps for an explicit scheme of the DRE (2.24) one can rewrite Equation (3.4) as

$$dt = \frac{dx^2}{6CD} \quad (3.5)$$

to obtain an expression that is dependent on the voxel size. Equation (3.5) was already implemented in the PSEM and was also implemented in the FDM in this work.

In the following, an optimal value for the new constant $C_n = 2nC$ was determined with the aim of (i) reducing the necessary number of time steps and thus the runtime of the simulation, and (ii) achieving voxel size-independent simulation results. Moreover, (iii) the value should be large enough so that the solution scheme remains numerically stable. After some testing (data not shown), the coefficients C_n , listed in Table 3.4, proved useful in satisfying these three conditions.

In particular, (iii) was satisfied with these values to avoid oscillations that occurred with the PSEM or the generation of invalid voxel values in the case of the FDM. It was also found that the minimum C_n is mostly independent of the voxel size for voxel sizes between 1 and 20 μm , so that dt in equation (3.5) is directly proportional to dx^2 .

Table 3.4. Minimum values of C_n determined for the TOM simulation that satisfy the CFL condition.

Dimension	Minimum C_n	
	2D	3D
FDM	5	7
PSEM	3	3

Two-stage termination condition for oxygen histogram simulations

The initial TOM 2013 version with a default voxel size of 20 μm and a simulation volume of 1 mm^3 was terminated after 400 time steps (iterations) when the solution approximately reached a steady state [27]. This low number of iterations, however, leads to inaccurate results for higher spatial resolution. A new termination condition was therefore introduced in this work that depends on (i) the oxygen tension changes of each voxel between two iteration steps and (ii) the changes of two oxygen histograms at different time steps, as shown in Figure 3.8.

Figure 3.8 shows that for condition (i) the maximum absolute percentage difference (MaxAPD) was used to detect a threshold to stop the TOM iterations and create a new histogram. The MaxAPD was then used to limit the maximum deviations of the individual oxygen tensions p_i at all grid points between the iteration steps t and $t + dt$:

$$\text{MaxAPD} = \max_i \left| 1 - \frac{p_i^t}{p_i^{t+dt}} \right| \quad (3.6)$$

If the MaxAPD falls below the threshold of 0.1 % between two consecutive time steps, the TOM script is terminated and an oxygen histogram is created for stage (ii).

In stage (ii), two consecutive histograms are compared using the earth mover's distance (EMD, see Section 2.2.4 for a description). When their EMD falls below 0.07 %, the algorithm terminates. If their difference is larger than the threshold or if there exists only one histogram, as in the first iteration of this loop, the loop continues until a new MaxAPD threshold, which is by 50 % lower than the previous threshold, is reached. Then the histograms are compared again until the simulation is finally terminated.

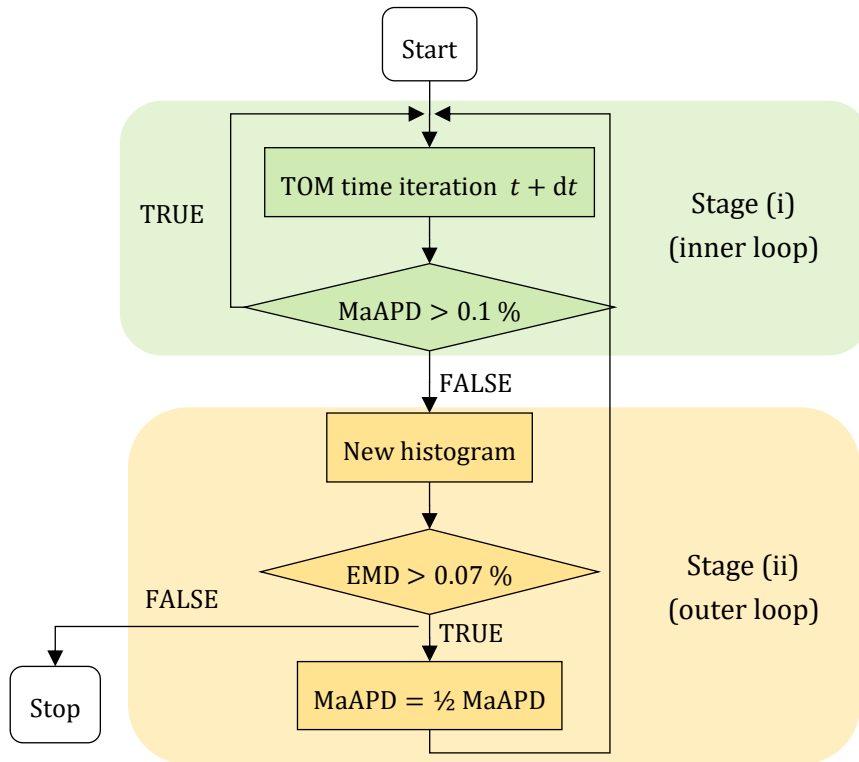


Figure 3.8. Two-stage termination condition for calculating a TOM histogram. The MaxAPD is, in this case, the maximum absolute deviation of all grid p_{O_2} values between two simulation grids generated in two consecutive time steps. The EMD is the earth mover's distance between two histograms. To fulfill the EMD condition, the total shift of the probabilities in the x-direction for the superposition of the two histograms must be smaller than 0.07 %. This shift refers e.g., to a shift of 1 % between two adjacent histogram bis for histograms with 16 bins as used in the TOM.

The reason for using this second condition is that the MaxAPD depends on many input parameters such as voxel size, simulation volume, absolute diffusion differences, and amount of vessel oxygenation. Moreover, a robust MaxAPD value without another condition could result in long simulation times with unnecessary high precision, especially for voxel sizes below 10 μm . Nevertheless, the MaxAPD is a useful condition because it is also sensitive to local changes in the oxygen tension, whereas other commonly used metrics such as the mean deviation may not detect local changes, e.g., in the case of a single capillary where the largest changes occur close to the capillary. Besides that, the second termination condition is useful, since it depends only on the change of the oxygen histograms, which are the most important output for the TOM, and ignores the dependencies of the MaxAPD.

3.1.4. TOM accuracy improvements for multi-capillary grids

After implementing the changes to achieve voxel-size independence of the TOM simulations based on single capillary simulations, the next step was to investigate the effect of these changes on the oxygen histograms of a multi-capillary grid. As shown in Figure 3.1, different authors reported substantially different oxygen histograms for comparable capillary architectures, most likely due to differences in (i) the algorithms, (ii) boundary conditions, and (iii) voxel size, which will be investigated below.

Simulation grid size

Ideally, the side length of the simulation volume in each dimension should be larger than twice the maximum diffusion distance to avoid interference of a capillary with itself in the case of periodic boundary conditions and to avoid boundary effects in the case of Dirichlet or von Neumann conditions. According to Figure 3.7 b), the maximum diffusion distance, defined here as the distance at which the p_{O_2} drops below 1 % of the initial capillary p_{O_2} of 40 mmHg, is less than 120 μm for the parameters used in this work. Thus, the side length of the simulation volume should be at least 240 μm in this case.

To investigate the dependence of the oxygen histograms on the simulation volume, 20 square 2D grids with side lengths ranging from 300 μm to 1500 μm in each dimension were simulated. A volume with a side length of 4000 μm was also added as a baseline to eliminate possible edge effects. A vascular fraction of 5 % was chosen for all simulations, as this is a representative value for the experimental values, which typically range from 1 % to 10 %. Furthermore, a vascular grid with a vascular fraction of 5 % leads to oxygen histograms with a maximum around 20 mmHg, i.e., in the middle of the histogram, so that changes due to different simulation parameters can be observed in both directions. The 3D simulations are expected to follow the same trends as the 2D cases, so simulations were restricted to 2D.

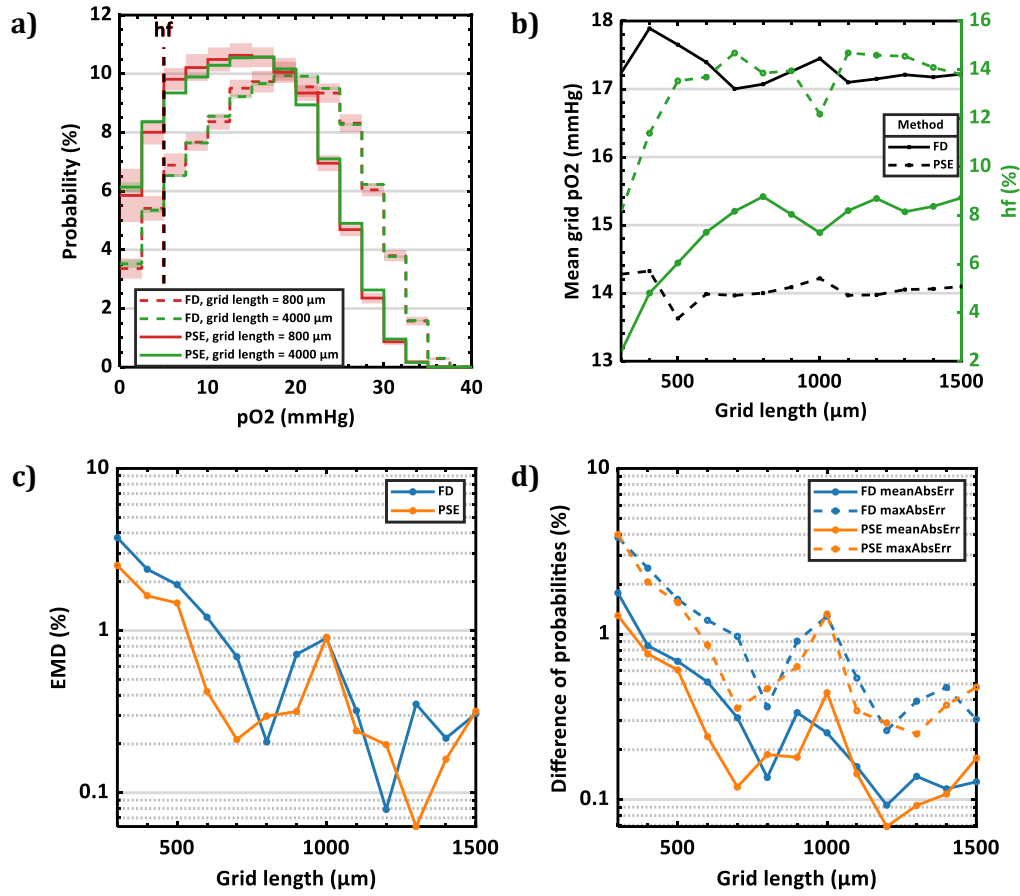


Figure 3.9. Dependence of the oxygen histograms on the simulation volume. a) Example histograms for two simulation volumes of 800 and 4000 μm side length and 20 μm voxel size. Both numerical methods for the same grid length agree quite well, as most bin values (mean of 20 simulations) agree within their standard error of the mean (SEM) limits. b) The mean pO_2 of the entire volume remains constant except for the smallest side length. In contrast, the hypoxic fraction (HF) falls drastically for grid lengths below 800 μm . c) The EMDs increase for grid lengths below 700 μm . For larger side lengths there are some fluctuations but no clear trends. d) Similarly, the mean and maximum deviation of the bins decrease up to 700 μm and remain essentially constant above this value.

Figure 3.9 shows that a side length of the simulation volume of at least 800 μm should be used to avoid boundary effects on (i) the hypoxic fraction (HF) and the mean pO_2 over the entire volume and (ii) the histogram metrics EMD, mean, and maximum absolute histogram bin difference.

Consequently, side lengths of at least 1500 μm should be used for quantitative simulations with higher accuracy since the variance of the histogram data continues to decrease. This variance is depicted in the histograms as the *standard error of the mean* (SEM) bands. Furthermore, the grid length of 1500 μm is preferable because the other quantities in Figure 3.9 also deviate only slightly from the baseline for this value.

The results for the default grid length of 1000 μm from Espinoza et al. [27] differ only slightly from those for 1500 μm , visible in a small increase in EMD (Figure 3.9 c)) and the difference of probabilities in (Figure 3.9 d)).

Voxel size

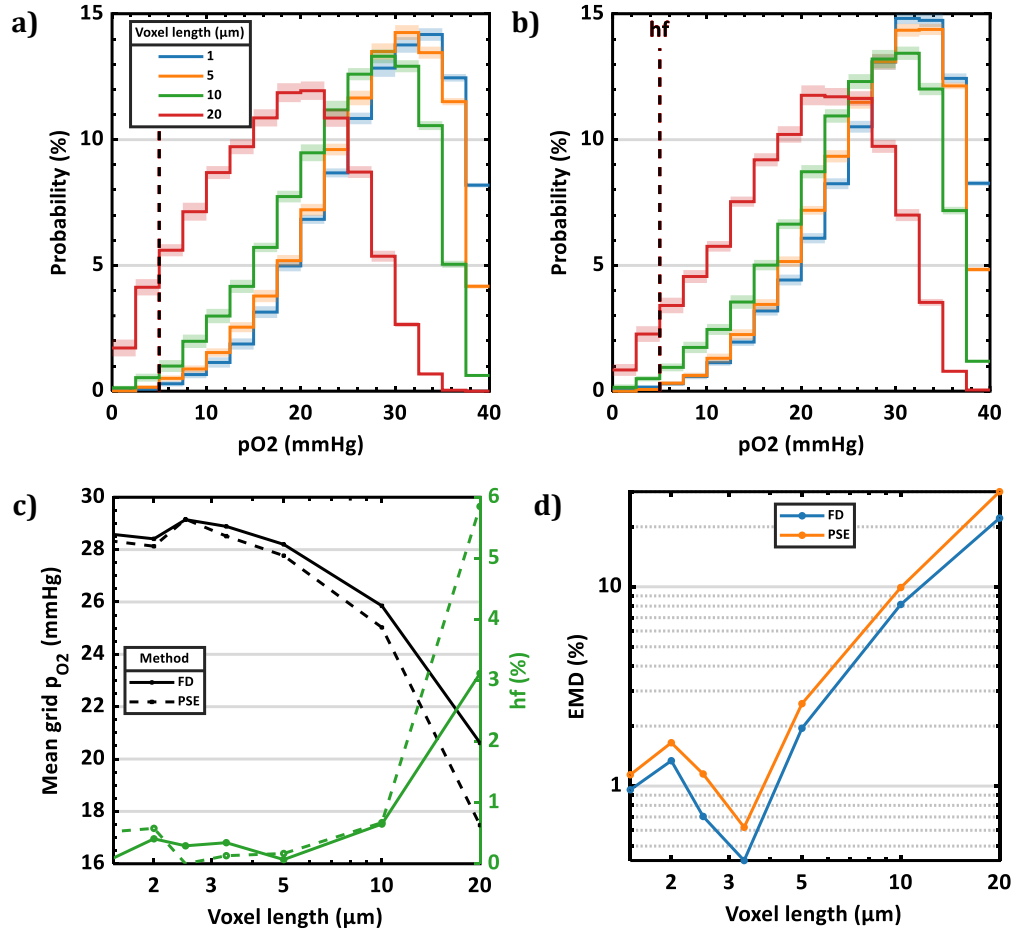


Figure 3.10. Dependence of histograms on voxel size evaluated for a VF of 5 % using capillaries with a square cross-section and a side length of 20 μm . Both PSEM (a) and FDM (b) show stable histogram shapes for voxel sizes at or below 5 μm . Thus, a quarter of the capillary side length or less appears to be necessary to avoid an underestimation of the well-oxygenated volumes in spatial proximity to the capillaries. c) Mean oxygenation and hypoxic fraction remain constant for voxel sizes less than 5 μm . d) For these voxel sizes, the EMD is less than 3 % for both methods, which is a factor of three less than for voxel sizes of 10 μm and more than a factor of four for 20 μm . MAD and MaxAD exhibited the same behavior (data not shown).

Based on the comparison of the simulated oxygen profiles of a single capillary with those of the analytical solution (Figure 3.4), the voxel size was expected to have the strongest influence on the numerical accuracy of the DRE solution. To investigate this,

the voxel size was varied between 1 μm , which is used as ground truth for this analysis, to 20 μm as used in Espinoza et al. [27].

Figure 3.10 illustrated a significant impact of the voxel size of square cross-section capillaries on the resulting oxygen histograms. The square cross-section was chosen because it ensures that the same capillary geometry is used for all voxel sizes. As the figure illustrates, only voxel sizes smaller than or equal to five micrometers led to comparable histograms and oxygenation parameters such as hypoxic fraction (HF) and mean p_{O_2} , so only these voxel sizes yielded stable results.

3.1.5. Optimized numerical TOM parameters

New parameter sets for standard and high precision simulations

Based on the findings in Section 3.1, the simulation parameters for the TOM were updated as listed in Table 3.5. In the following, PSEM and FDM were compared using these updated parameters to check if they led to comparable histograms for both precision settings, i.e., standard and precision.

Table 3.5. Revised parameters for TOM simulations

Parameter	Value		
	Old	Standard precision	High precision
Voxel size	20 μm	5 μm	2 μm
Side length of cubic simulation volume	1000 μm	800 μm	1500 μm
Numerical method	PSEM	FDM**	PSEM & FDM
Termination condition	400 iterations	Two stages: • MaxAPD (volume) $\leq 0.1 \%$ • EMD (histogram) $\leq 0.07 \%$	MaAPE $\leq 10^{-4}\%$ *

*If the exact p_{O_2} of single grid points is important. Otherwise, the two-stage stop condition (Figure 3.8) from the standard approach may be used.

**The parameters used here for the FDM, as the new standard, can be applied equivalently to the PSEM.

Influence of the selected parameter set on the numerical results

Figure 3.11, Table 3.6, and Table 3.7 show that the difference between standard and high precision settings was smaller when using the FDM in comparison to the PSEM and that both methods led to comparable histograms with only minor differences. Since the largest histogram deviations in Figure 3.11 b) occurred for bins larger than 18 mmHg, they are less relevant to the cellular radiosensitivity changes which are dominated by the leftmost bins.

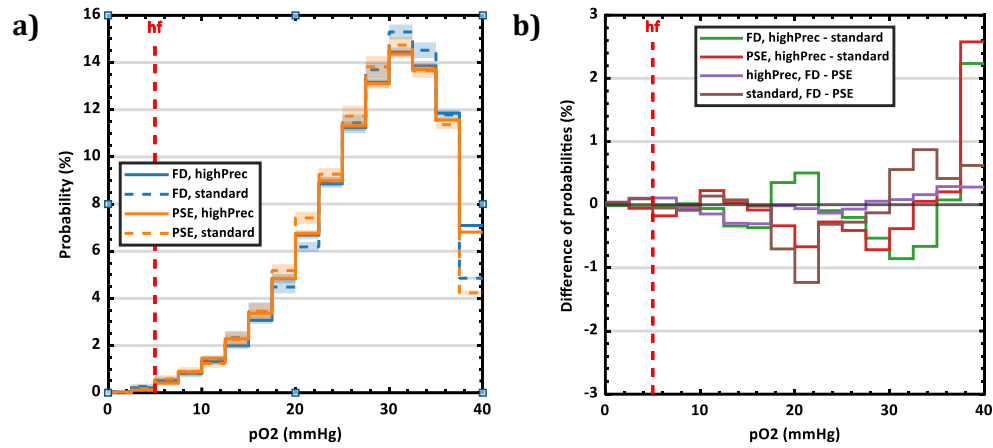


Figure 3.11. Dependence of the histograms on the numerical method for multi-capillary settings. The simulations were performed with the standard and high-precision parameters of both numerical methods (see Table 3.5). In panels a) and b) both methods have only slightly higher medium oxygenation levels for the standard as compared to the high precision settings.

This finding is also reflected in the dose enhancement ratios (DER) in Table 3.6, which are well comparable for all four simulated cases. As shown in Figure 3.7, the accuracy of FDM and PSEM were also comparable when benchmarking against the analytical solution.

Table 3.6. Quantitative comparison of the histograms in Figure 3.11. The difference between standard and high precision for the same method was larger than the difference between both methods for high precision. No similar finding was evident for the standard precision.

Parameter	Standard vs. high precision		PSEM vs. FDM	
	PSEM	FDM	Standard precision	High precision
EMD (%)	1.05	0.63	0.90	0.44
MAPD (%)	0.39	0.40	0.35	0.13
MaxAPD (%)	2.58	2.24	1.23	0.30
DER	1.00	1.00	1.00	1.00

- *Abbreviations:* EMD = earth mover's distance, MAPD = mean absolute percentage deviation, MaxAPD = maximum absolute percentage deviation, DER = dose enhancement ratio. For a complete description of the parameters, see Section 2.2.4.

Furthermore, Table 3.7 shows that the oxygenation status of the simulated volume is only slightly affected by the different parameter combinations.

Table 3.7. Quantitative comparison of the simulated volumes, on which the histograms shown in Figure 3.11 are based. The mean p_{O_2} of the simulation volume is only slightly affected by precision or method. The HF differs slightly more between different methods than with different precisions of the same method.

Parameter	Standard precision		High precision	
	PSEM	FDM	PSEM	FDM
Mean grid p_{O_2} (mmHg)	27.9	28.2	28.3	28.4
HF (%)	0.17	0.30	0.14	0.24

The FDM was chosen as the new standard method in Table 3.5 because of its higher computational speed due to the smaller number of nearest neighbor calculations (in a distance of one voxel) for each time step compared to the PSEM (distance of 5 voxels). This resulted in a significant speedup for a multi-capillary setting as introduced in Espinoza et al. [27]: The FDM with standard precision was 18 times faster for a 2D simulation (12 ms runtime) and 130 times faster for a 3D setting (200 ms runtime).

For special simulations, where e.g., the oxygen tensions of the individual voxels are analyzed or the absolute histogram values are important, the high-precision settings should be used and in addition, the results of the two numeric methods should be compared. The runtime for high-precision settings is almost a factor of four shorter for the FDM (2.6 s) than for the PSEM (9.4 s) with the same simulation settings and comparable EMD.

Consequences of the parameter updates for the oxygen histograms

Figure 3.12 indicates that the parameter updates of the TOM 2021 version resulted in better tissue oxygenation compared to the TOM 2013 version. Thus, the calculation of the oxygen-dependent radiation-induced cell kill using the updated oxygen histograms would result in a lower survival fraction. This effect was calculated as an example for the capillary grid in Figure 3.12, which resulted in a DER of 1.22 at a SF of 1 %. Consequently, with the original TOM 2013 version, a 22 % higher single-fraction dose would be required to achieve the same 1 % SF as with the TOM 2021 version.

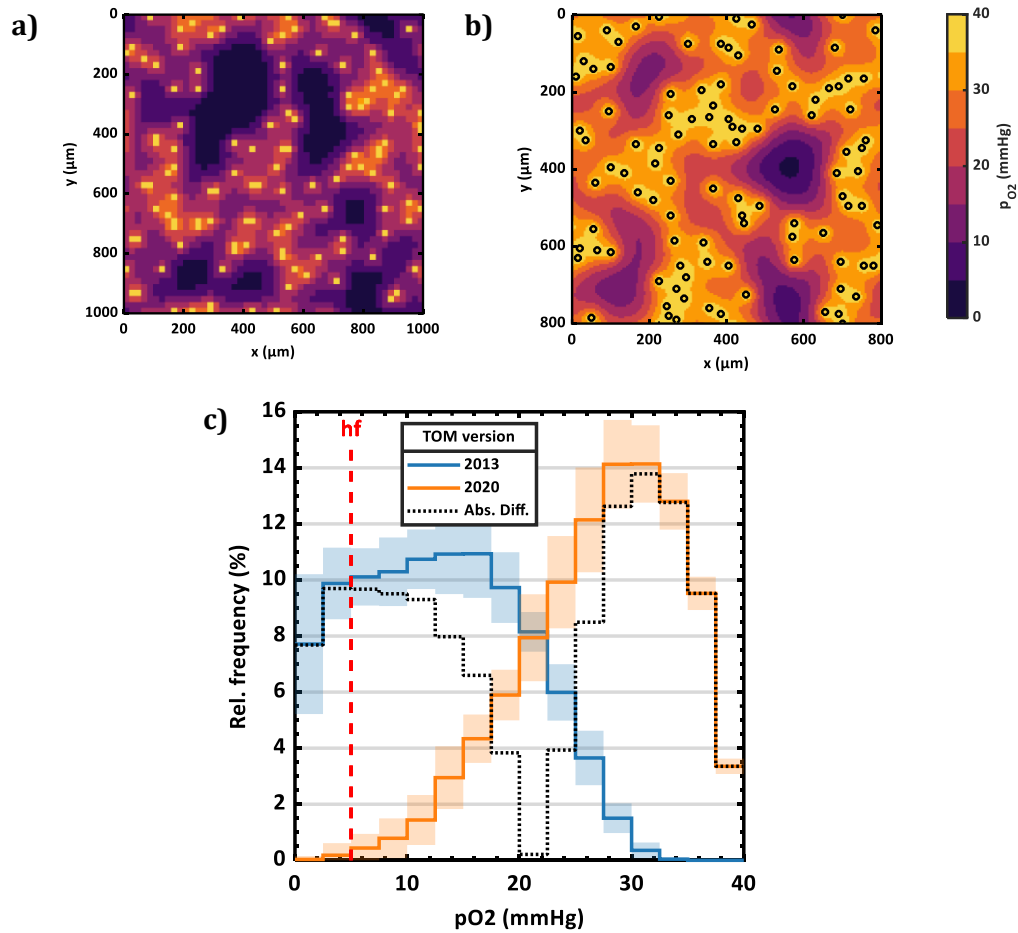


Figure 3.12. Consequences of the parameter updates for the oxygen histograms for an exemplary capillary grid with a vascular fraction of 5 % and capillary diameters of 20 μm . The oxygen histograms show the means $\pm$ standard deviations of 20 independent simulations. The 2D oxygen simulations were performed a) with the original TOM version from 2013 [27] and b) with the TOM 2021 version with the standard parameters from Table 3.5. Panel c) shows a right-shift of the oxygen histograms calculated with the TOM 2021 compared to the TOM 2013. The quantitative comparison of the two curves shows a MAD of 8 %, a MaxAD of 14 %, an EMD of 38 %, and a DER of 1.22 at a 1 % SF. For a parameter description see Section 2.2.4.

3.2. Empirical characterization of the tumor vasculature

The tissue samples were analyzed using the workflow in Section 2.3.4, where the 3D datasets were split into several cubic subvolumes. This partitioning was performed for two reasons: Time-efficient analysis of the data sets and comparison of different tumor sub-volumes. The side length of 800 μm for each cubic subvolume was selected for two reasons: (i) This side length was determined in Section 3.1.4 to be the smallest size for a TOM simulation grid to still avoid partial volume effects. (ii) The resulting

volume is approximately the size of an MRI voxel and thus could help in translating the microscopic results to a macroscopic area.

In this chapter, the results of the tissue analysis are presented, with the first section focusing on vascular parameters that are comparable with histological (2D) sections. Then, the conventional vascular parameters, which are only measurable in 3D, are analyzed and in the last section. the vascular network is investigated using a graph-theoretic approach. Thus, an empirical investigation of the vascular network is performed and in a second step, the tumor oxygenation and hypoxia are simulated based on the vascular architecture alone.

3.2.1. Comparison of 2D histological and 3D LSFM quantities

The comparison of 2D histological and 3D LSFM data was summarized in Table 3.8 and discussed in the following sections. A description of the parameter calculations can be found in Section 2.3.4. The LSFM data analysis was more time-consuming compared to the acquisition of the histological slices, requiring, for example, four weeks of sample preparation, one week of imaging, and four weeks of post-processing. Thus, a considerably lower *number of animals* of only 12 % of the histological cohort was analyzed.

Figure 3.11 a) shows, that the *mean radii* $\bar{r}$ for the SAS tumor are consistent between methods, while the UT-SCC-14 has significantly lower medians for the LSFM measurements. Table 3.8 shows comparable trends. Besides that, the mean rat brain radii obtained in this work agree well with the mouse brain data of Hahn et al. (2019) [164] with a deviation of 8 % in Table 3.8.

The *Median vessel radii* $\tilde{r}$, were used as a second radius quantity for two reasons: (i) The vessel radii typically exhibit a log-normal distribution [37], [164], so the median gives additional information about the shape of the distribution. (ii) The histological data obtained by J. Müller in his Ph.D. project [114] also included this quantity. Table 3.8. shows that the median vessel radii are at least 19% higher for the 3D LSFM as compared to the 2D histological sections, being 19 to 47 % higher for the UT-SCC-14, and 62 to 96 % higher for the SAS arms.

The mean and median vessel radii of all specimens are in a comparable range of [3.2, 6.7] μm and [2.6, 5.4] μm , respectively. For the LSFM method, the mean values are slightly larger than the median values for the same group with a maximum deviation of 0.5 μm , whereas the mean values of the histology are almost twice as large as the median values (see also Figure 3.13). Fractionation has little effect on vessel radii, with a small trend towards larger mean and median radii with higher doses for LSFM data from SAS and UT-SCC-14 (see also Figure 3.13 b)).

The *mean vascular fraction* $\overline{VF}$ in Table 3.8 is comparable for mouse and rat brains showing a 15 % difference. However, the rat brain samples show a large variance between the two samples, which likely contributes to the deviation from the mouse brain data from [164]. Besides that, the comparison between histology and LSFM also shows similar values with a larger spread for the LSFM data (in Figure 3.13 c)). However, an outlier is the SAS tumor control group measured by LSFM, which is more than a factor of two smaller than the histology data. Insufficient staining of the LSFM samples or insufficient segmentation could play a role here.

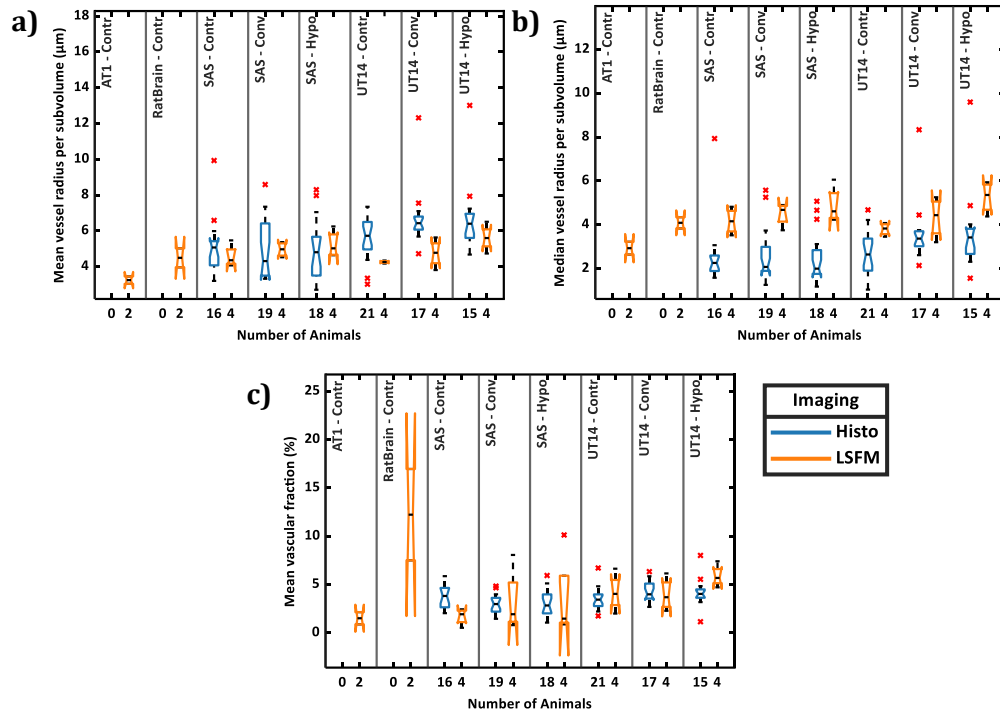


Figure 3.13. Cumulative quantities for the animals, listed in Table 3.8. a) Radius averaged consecutively over each vessel, over the partial volume (LSFM) or slice (histology), and over the respective animal per group. b) Radius averaged over each vessel, followed by determination of the median over the partial volume (LSFM) or slice (histology), and averaging over the respective animal and over the group. c) Vascular fractions of the subvolumes averaged consecutively per animal and over the group. *Note:* The notches of the boxplots indicate the 95 % CI for the medians. Besides that, the whiskers show the 95 % CI for covering all data, i.e., the 2σ -interval for a normally distributed variable.

Table 3.8. Geometric vessel characteristics of the brain tissue and the tumor samples. The data are presented as mean $\pm$ standard deviation of each quantity per group. Grouping refers to all animals of the selected tissue type treated with the same fractionation schedule. For the *LSFM data sets*, the radii were calculated along the vessel center line of each vessel and then averaged for each vessel. Then the average of all vessels ($\bar{r}$) or the median of all vessels ($\tilde{r}$) was calculated for each subvolume, followed by an averaging for each animal. Lastly, the group average was calculated, as tabulated here. For the mean vascular fraction $\overline{VF}$ the same procedure as for the radius parameters was used except for the first step (averaging for each vessel). For the *histology datasets*, radii were calculated using the minor half axis of the fit ellipse fitted to each vessel cross-section of the slice. Then the average of all vessel radii per slice ($\bar{r}$) or the median of all vessel radii per slice ($\tilde{r}$) was calculated for each slice, followed by an averaging for each animal. Lastly, the group average was calculated, as tabulated here. For the mean vascular fraction $\overline{VF}$ the same procedure as for the radius parameters was used except for the first step (averaging for each vessel).

Tissue type	Image type	Fractionat.	N	$\bar{r}$ (μm)	$\tilde{r}$ (μm)	$\overline{VF}$ (%)
<i>Normal tissue</i>						
Brain (mouse)*		Untreated	6	$4.9^{+1.7}_{-1.2}$ **	/	10.3 ± 3.7
Brain (rat)		Untreated	2	4.5 ± 0.8	4.1 ± 0.4	12.2 ± 6.7
<i>Tumors</i>						
AT1 (rat)	LSFM	Untreated	2	3.2 ± 0.3	2.9 ± 0.4	1.5 ± 0.9
SAS (mouse)	Histo	Control	16	5.1 ± 1.6	2.6 ± 1.5	3.7 ± 1.2
		Convent.	19	5.1 ± 1.6	2.7 ± 1.2	3.0 ± 1.0
		Hypofract.	18	4.9 ± 1.7	2.5 ± 1.1	3.1 ± 1.3
	LSFM	Control	4	4.6 ± 0.6	4.2 ± 0.6	1.7 ± 0.8
		Convent.	4	5.0 ± 0.4	4.5 ± 0.5	3.2 ± 3.3
		Hypofract.	4	5.2 ± 0.8	4.9 ± 0.8	3.5 ± 4.4
	Histo	Control	21	5.7 ± 1.2	2.7 ± 1.0	3.5 ± 1.1
		Convent.	17	6.7 ± 1.6	3.6 ± 1.3	4.3 ± 1.1
		Hypofract.	15	6.7 ± 2.0	3.6 ± 1.8	4.1 ± 1.4
	LSFM	Control	4	4.3 ± 0.1	3.8 ± 0.3	4.2 ± 1.9
		Convent.	4	4.7 ± 0.8	4.3 ± 0.9	4.0 ± 1.7
		Hypofract.	4	5.6 ± 0.7	5.3 ± 0.7	5.9 ± 1.1

Abbreviations: Histo = Histology, LSFM = light-sheet fluorescence microscopy, N = number of animals, $\bar{r}$ = radius averaged over the subvolumes (LSFM) or slice (histology), $\tilde{r}$ = median of the radius over the subvolumes (LSFM) or slice (histology), $\overline{VF}$ = vascular fractions averaged over the subvolumes (LSFM) or slices (histology).

*Data from [164].

**Average directed deviations for log-normal distributed data from [164].

3.2.2. Conventional 3D vascular parameters

Table 3.9. Conventional vascular network parameters for the brain and tumor samples. The data are presented as mean $\pm$ standard deviation of each quantity per group. Grouping refers to all animals of the selected tissue type treated with the same fractionation schedule. For the *density parameters* and *distance measures*, the average of all vessels or distances was calculated for each subvolume, followed by an averaging for each animal. Lastly, the group average, as tabulated here, was calculated. Each *branching parameter* was first calculated for each vessel (e.g., $l_{\max,B,V}$) or averaged over each vessel (e.g., $\bar{l}_{B,V}$). Then the average of all vessels was calculated for each subvolume, followed by an averaging for each animal. Lastly, the group average was calculated, as tabulated here.

Animal	Rat		Mouse						
Tissue type	AT1 tumor	Brain	Brain*	SAS tumor	UT-SCC-14 tumor				
Fractionat.	Untreated	Untreated	Un-treated	Control	Convent.	Hypofract.	Control	Convent.	Hypofract.
V_{tot} (mm ³)	20 $\pm$ 10	90 $\pm$ 110	/	20 $\pm$ 20	50 $\pm$ 30	20 $\pm$ 20	20 $\pm$ 30	40 $\pm$ 20	20 $\pm$ 10
<i>Density parameters</i>									
$\bar{\rho}_B$ (10 ³ mm ⁻³)	4.1 $\pm$ 1.2	7.3 $\pm$ 0.4	/	1.2 $\pm$ 0.6	1.1 $\pm$ 0.7	1.3 $\pm$ 0.9	2.5 $\pm$ 1.4	2.1 $\pm$ 0.9	2.0 $\pm$ 0.5
$\bar{\rho}_V$ (mm ⁻³)	2300 $\pm$ 30	640 $\pm$ 90	/	510 $\pm$ 200	400 $\pm$ 150	420 $\pm$ 210	540 $\pm$ 370	600 $\pm$ 270	540 $\pm$ 240
$\bar{\rho}_{VL}$ (mm/mm ³)	141 $\pm$ 67	400 $\pm$ 53	980 $\pm$ 99	47 $\pm$ 25	52 $\pm$ 38	57 $\pm$ 52	108 $\pm$ 57	90 $\pm$ 34	95 $\pm$ 25
<i>Branching parameters</i>									
$\bar{l}_{B,V}$ (μ m)	27.2 $\pm$ 0.1	31.7 $\pm$ 1.5	/	25.7 $\pm$ 5.1	32.9 $\pm$ 6.2	33.1 $\pm$ 12.5	28.7 $\pm$ 9.1	26.6 $\pm$ 6.5	29.3 $\pm$ 5.0
$l_{\max,B,V}$ (μ m)	30.8 $\pm$ 0.8	39.7 $\pm$ 3.5	/	33.9 $\pm$ 6.3	42.7 $\pm$ 6.8	45.6 $\pm$ 19.1	42.9 $\pm$ 14.8	36.7 $\pm$ 8.9	39.9 $\pm$ 8.6
$\bar{l}_B$ (μ m)	33.3 $\pm$ 4.0	54.2 $\pm$ 5.6	19 ⁺¹⁵ ₋₉	40.5 $\pm$ 5.9	45.2 $\pm$ 4.1	47.3 $\pm$ 14.9	43.1 $\pm$ 4.9	41.7 $\pm$ 6.2	47.1 $\pm$ 5.5
$\bar{\tau}$	1.15 $\pm$ 0.03	1.26 $\pm$ 0.02	/	1.24 $\pm$ 0.01	1.25 $\pm$ 0.05	1.25 $\pm$ 0.03	1.30 $\pm$ 0.05	1.27 $\pm$ 0.02	1.27 $\pm$ 0.02
$\tilde{\tau}$	1.06 $\pm$ 0.03	1.16 $\pm$ 0.01	1.07	1.15 $\pm$ 0.03	1.13 $\pm$ 0.03	1.13 $\pm$ 0.03	1.16 $\pm$ 0.05	1.14 $\pm$ 0.01	1.15 $\pm$ 0.03
τ_{95}	1.62 $\pm$ 0.08	1.79 $\pm$ 0.15	1.45	1.81 $\pm$ 0.06	1.84 $\pm$ 0.14	1.83 $\pm$ 0.06	2.01 $\pm$ 0.12	1.93 $\pm$ 0.05	1.88 $\pm$ 0.03
$\bar{N}_{B,V}$	1.7 $\pm$ 0.4	22.3 $\pm$ 14.5	/	2.2 $\pm$ 0.3	2.7 $\pm$ 1.2	3.5 $\pm$ 3.0	5.0 $\pm$ 0.9	3.6 $\pm$ 1.3	4.4 $\pm$ 1.3
$\bar{N}_{B,N}$	1.50 $\pm$ 0.15	2.48 $\pm$ 0.21	/	1.76 $\pm$ 0.14	1.78 $\pm$ 0.19	1.84 $\pm$ 0.32	2.23 $\pm$ 0.05	2.02 $\pm$ 0.15	2.06 $\pm$ 0.13
<i>Distance measures</i>									
$\bar{d}_{\min,T,V}$ (μ m)	52 $\pm$ 10	38 $\pm$ 20	/	99 $\pm$ 40	90 $\pm$ 25	92 $\pm$ 39	84 $\pm$ 34	73 $\pm$ 11	64 $\pm$ 10
$\bar{d}_{\min,V,V}$ (μ m)	50.7 $\pm$ 1.7	84.9 $\pm$ 8.2	/	96.3 $\pm$ 17.6	96.7 $\pm$ 13.4	97.7 $\pm$ 22.1	81.2 $\pm$ 16.6	80.9 $\pm$ 12.9	86.6 $\pm$ 14.0

Abbreviations: V_{tot} = total evaluated volume, **Density parameters:** $\bar{\rho}_B$ = branch density (number of branches per volume), $\bar{\rho}_V$ = vessel density (number of vessels per volume), $\bar{\rho}_{VL}$ = total vessel length density (total vessel length per volume), **Branching parameters:** $\bar{l}_{B,V}$ = mean branch length per vessel, $l_{\max,B,V}$ = maximum branch length per vessel, $\bar{l}_B$ = mean branch length in the subvolume, $\bar{\tau}$ = mean tortuosity, $\tilde{\tau}$ = median tortuosity, τ_{95} = 95 % quantile of the tortuosity, $\bar{N}_{B,V}$ = mean number of branches per vessel, $\bar{N}_{B,N}$ = mean number of branches per node, **Distance measures:** $\bar{d}_{\min,T,V}$ = minimum distances of 10⁵ randomly selected tissue voxels to the next vessel center, $\bar{d}_{\min,V,V}$ = minimum inter-vessel distance between vessel centers.

*Data from [164].

Only the LSM datasets were used for this 3D characterization of the vascular network. Thereby, the characterization with the conventional vascular parameters was based on the vessel skeleton, i.e., the vessel centerlines. The evaluated 3D parameters were summarized in Table 3.9. A description of the parameter calculations can be found in Section 2.3.4.

The *total volumes* V_{tot} that were available for analysis ranged between 20 and 50 mm³ for the tumors, with more than twice the volume available for the conventional fractionations compared with the others. The two rat brain samples showed the largest variance and the largest absolute values, most likely due to staining differences.

Density parameters

The *density of branches* $\bar{\rho}_B$, i.e., the number of branches per volume, of the rat brain was significantly higher than that of the tumors. As a result, the AT1 tumor showed a 43 % lower density and the UT-SCC-14 tumor had at least a 66 % reduced density. Besides that, the $\bar{\rho}_B$ of UT-SCC-14 further decreased for the hypofractionated schedule, whereas the SAS tumor had at least an 82 % lower $\bar{\rho}_B$ that did not differ between both fractionation schemes. In contrast, the AT1 tumor exhibited the highest *density of vessels* $\bar{\rho}_V$, which was 3.6-times higher than in the rat brain, while the remaining samples showed values like the brain. Additionally, the SAS tumor showed a slight decrease in density with increasing fractional dose.

The *total vessel density* $\bar{\rho}_V$, i.e., the number of vessels in a volume, shows similar trends as the density of the branches: The brain had the highest $\bar{\rho}_V$, which was threefold higher than that of AT1 tumors, at least fourfold higher than that of UT-SCC-14 tumors, and at least sevenfold higher than that of SAS tumors. The mouse brain data from [164] showed 2.5-times higher values than the rat brain, which could originate from different vascular properties of the animals, but also to different sample qualities. Besides that, a slight effect of the irradiation could be seen for the tumors: The irradiated SAS tumors exhibited up to 21 % higher length densities, while the length density in UT-SCC-14 after irradiation decreased up to 17 % compared to the control group. However, it should be noted that the variances are also large so that the observed trend could also be due to the small number of animals.

In contrast to these findings are the results for the *mean vessel length density* $\bar{\rho}_{VL}$: This quantity is defined as the total length of all vessels in a subvolume divided by that volume, and is thus a vessel-radius-independent alternative to the vascular fraction. The $\bar{\rho}_{VL}$ slightly decreased in SAS tumors after irradiation and increased in UT-SCC-14 tumors (see Figure 3.14 a)). Additionally, the magnitude of the differences in $\bar{\rho}_{VL}$ of the different tissue samples compared with the rat brain in Table 3.9 was similar as the deviations of the $\overline{VF}$ in Table 3.8: The $\bar{\rho}_{VL}$ were eight times smaller for the AT1

tumors, at least three times smaller for the SAS tumors and at least two times smaller for the UT-SCC-14 tumors.

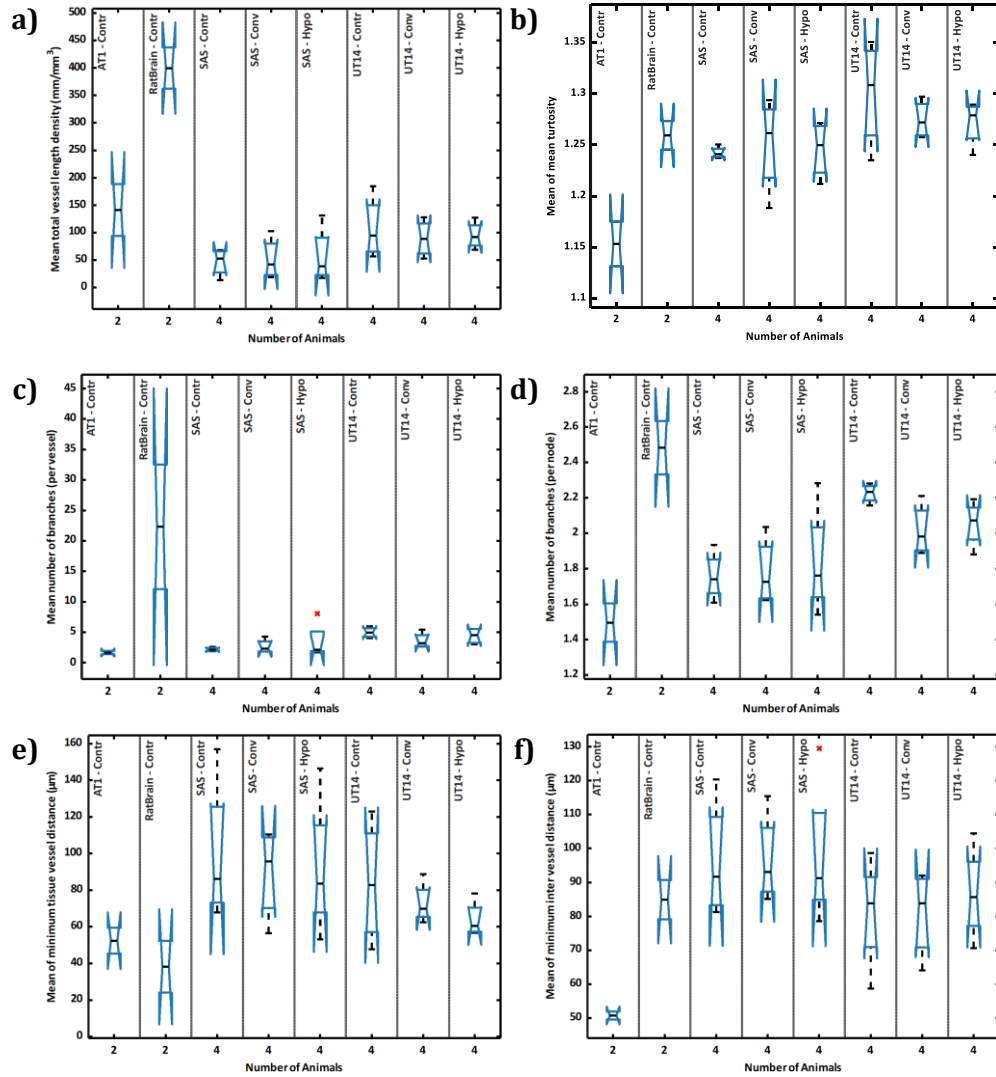


Figure 3.14. Density parameters pooled for all animals of the selected tissue type treated with the same fractionated schedule. a) Total vessel length density, b) mean tortuosity of all branches, c) mean number of branches per vessel, d) mean number of branches per node, e) minimum tissue-to-vessel distance for 10^5 randomly selected measurement points, f) minimum inter-vessel distance between all vessels in a subvolume. *Note:* The notches of the boxplots indicate the 95 % CI for the medians. Besides that, the whiskers show the 95 % CI for covering all data, i.e., the 2σ -interval for a normally distributed variable.

Branching parameters

The *length $\bar{l}_{B,V}$ of all branches averaged for each coherent vessel* varied from 25.7 to 33.1 μm for the whole set of tissue samples. The mouse samples showed at least 3-fold higher variance than the rat samples, which could indicate a large intra-tumoral heterogeneity. The maximum length of all branches per vessel $l_{\text{max},B,V}$ showed similar trends for the variances.

The *length $\bar{l}_B$ of all branches averaged over each subvolume* indicated significant differences between tissue types. The rat brain exhibited the highest $\bar{l}_B$ with $(54.2 \pm 5.6) \mu\text{m}$, the AT1 tumors had 39 % lower values, and both the SAS and the UT-SCC-14 tumors had at least 13 % shorter branches. Besides that, the SAS tumors showed a slight trend towards larger $\bar{l}_B$ at higher doses, while no clear trend was evident for the UT-SCC-14 tumor. Compared to the mouse brain data from [164], the rat brain showed an almost 3-fold higher $\bar{l}_B$ with a deviation of seven standard deviations.

The *tortuosity* describes the curvature of each branch, i.e., its length divided by the Euclidean distance of its two endpoints. The *mean tortuosity $\bar{\tau}$* (of all branches per subvolume) is almost identical for all samples. The only exceptions were the AT1 tumors, which had a 9 % reduced $\bar{\tau}$ compared to the other tissue types and thus exhibited more straight-formed branches (see also Figure 3.14 b). However, the medians of the boxplots in Figure 3.14 b) indicate larger differences than the mean values from Table 3.9. The *median tortuosity $\tilde{\tau}$* and the *95 % quantile τ_{95}* show similar trends as the mean tortuosity. Thereby, the value for the AT1 tumors in both cases was 9 % lower compared to the rat brains.

The mouse brain data from [164] exhibited a trend toward lower values as compared to the rat brain: The deviations were larger than two standard deviations and 8 % and 19 % lower values for median and 95 % quantile, respectively.

The *mean number of branches per vessel $\bar{N}_{B,V}$* varied considerably between tissue samples, with the rat brain having a significantly higher number than the tumors. Additionally, the rat brain had the largest standard deviation that was up to a factor of 5 higher than the standard deviation of the tumors (see also Figure 3.14 c)). Besides that, the AT1 tumor had the largest deviation from the rat brain with a 13-fold smaller value, followed by the SAS tumor with at least six-fold smaller values and UT-SCC-14 with four-fold smaller values.

The *mean number of branches $\bar{N}_{B,N}$ connected at one node* was significantly lower in the tumors than in rat brain: The $\bar{N}_{B,N}$ were reduced by at least 10 % (UT-SCC-14 tumor), at least 26 % (SAS tumor), and 40% (AT1 tumor). Besides that, the $\bar{N}_{B,N}$ in the SAS tumors showed almost no dose-dependence with less than 5 % change after irradiation, whereas irradiation led to a decrease of at least 8 % for the UT-SCC-14 tumors.

Distance measures

The distance measures proved to be useful to characterize the spatial vessel distribution in the volume, which may allow some predictions of tissue oxygenation. The vascular fraction alone would not be sufficient for this purpose, as it only provides information about the vascular volume, not the vascular distribution in the sample.

The *minimum tissue-to-vessel distance* (TVD) describes the Euclidean distance of a tissue voxel to the nearest voxel of the vessel center line. The average minimum TVD ($\bar{d}_{\min,T,V}$) was determined as the distances for 10^5 randomly selected tissue voxels to their closest vessels that were averaged for each subvolume. The smallest distances were found in the rat brain, which would indicate the highest oxygenation of the tissue if all vessels were assumed to be functional. The AT1 tumors had a 37 % higher mean $d_{\min,T,V}$ than the rat brains, followed by the UT-SCC-14 tumors with 68 % and SAS tumors with 240 % higher values (see also Figure 3.14 d)).

The *minimum inter-vessel distance* (IVD) is defined as the shortest Euclidean distance from the center line of one vessel to the center line of the nearest vessel. The IVD is averaged over all vessels in a subvolume ($\bar{d}_{\min,V,V}$), and shows a different order for the deviations from the rat brain than the $d_{\min,T,V}$: In the case of the $\bar{d}_{\min,V,V}$, the AT1 tumor shows the smallest mean value (40 % less than the brain) and the smallest (single-digit) variance. In contrast, the brain and the other tumors have variances in the two-digit range (see also Figure 3.14 e)). While the UT-SCC-14 distances are within the one-standard-deviation of the brain values, the SAS tumor exhibits deviations of at least 13 % higher values than the rat brain.

3.2.3. Advanced 3D properties of the vascular networks

In addition to the conventional 3D vascular parameters, a graph-theoretic approach was used to examine the network properties of the vasculature. A description of the parameter calculations can be found in Section 2.3.4. The characterization using the network features was summarized in Table 3.10 and discussed in the following paragraphs. Thereby, the focus lies on (i) node parameters, characterizing density and branching of the individual nodes, (ii) network size parameters, referring to the spatial extent of the network, and (iii) network connectivity parameters, denoting the transport properties of the network.

Table 3.10. Network parameters of the largest vessel structures that have at least 10 connected edges (branches). The data are presented as mean $\pm$ standard deviation of each quantity per group. Grouping refers to all animals of the selected tissue type treated with the same fractionation schedule. For the *node parameters*, each quantity was calculated directly for each subvolume ($\bar{\rho}_B, \bar{\rho}_E, \bar{f}_E$) or as the average of all nodes in a subvolume ($\bar{k}$ and $k_{\max}$). Then, the values were averaged for each animal, and finally, the group average was calculated, as tabulated here. For the *graph size parameters*, each quantity was calculated directly for each subvolume ($\bar{\rho}_G, \bar{\rho}_{LG}, \bar{f}_{SG}, S$) or as the average of all graphs in a subvolume ($\bar{L}, \bar{R}, \bar{D}$). Then, the values were averaged for each animal, and finally, the group average was calculated, as tabulated here. For the *network connectivity parameters*, each quantity was calculated for each graph ($\bar{E}_{\text{loc}}$ and $\bar{E}_{\text{glob}}$) and then averaged over all graphs in a subvolume. the characteristic path length $\bar{C}$, in contrast, was calculated for each node, averaged for each graph, and then averaged for each subvolume. Then, these network connectivity parameters were averaged for each animal, and finally, the group average was calculated, as tabulated here. The calculated quantities based on the weighted graphs are recognizable by the dimensional unit of length (micrometer), while the dimensionless quantities were calculated from the unweighted graphs.

Animal	Rat		Mouse			UT-SCC-14			
Tissue type	AT1	Brain	Brain*	SAS					
Fractionat.	Untreated	Untreated	Untreated	Control	Convent.	Hypofract.	Control	Convent.	Hypofract.
<i>Node parameters</i>									
$\bar{\rho}_B$ (10^3mm^{-3})	1.09 $\pm$ 0.39	2.40 $\pm$ 0.48	/	0.36 $\pm$ 0.20	0.33 $\pm$ 0.38	0.44 $\pm$ 0.57	0.80 $\pm$ 0.33	0.92 $\pm$ 0.25	1.06 $\pm$ 0.39
$\bar{\rho}_E$ (10^3mm^{-3})	1.12 $\pm$ 0.40	1.72 $\pm$ 0.50	/	0.38 $\pm$ 0.20	0.34 $\pm$ 0.36	0.43 $\pm$ 0.52	0.82 $\pm$ 0.36	0.87 $\pm$ 0.26	0.89 $\pm$ 0.17
$\bar{f}_E$ (%)	53.9 $\pm$ 0.6	46.4 $\pm$ 2.3	11 $\pm$ 2	53.7 $\pm$ 1.4	54.6 $\pm$ 3.3	54.0 $\pm$ 3.1	51.3 $\pm$ 1.3	49.9 $\pm$ 0.6	48.5 $\pm$ 3.1
$\bar{k}$	1.90 $\pm$ 0.00	2.01 $\pm$ 0.06	3.24 $\pm$ 0.11	1.91 $\pm$ 0.02	1.91 $\pm$ 0.04	1.92 $\pm$ 0.04	1.95 $\pm$ 0.02	1.95 $\pm$ 0.01	1.99 $\pm$ 0.05
$k_{\max}$	4.06 $\pm$ 0.18	4.70 $\pm$ 0.51	20.7 $\pm$ 4.1	3.97 $\pm$ 0.17	3.96 $\pm$ 0.42	4.10 $\pm$ 0.52	4.30 $\pm$ 0.23	4.31 $\pm$ 0.10	4.63 $\pm$ 0.36
<i>Graph size parameters**</i>									
$\bar{\rho}_G$ (mm^{-3})	1780 $\pm$ 350	610 $\pm$ 140	/	390 $\pm$ 210	310 $\pm$ 80	270 $\pm$ 60	370 $\pm$ 250	450 $\pm$ 200	330 $\pm$ 160
$\bar{\rho}_{LG}$ (mm^{-3})	27.0 $\pm$ 6.3	20.0 $\pm$ 11.0	/	12.0 $\pm$ 5.0	10.0 $\pm$ 2.0	10.0 $\pm$ 2.0	22.0 $\pm$ 13.0	17.0 $\pm$ 7.0	11.0 $\pm$ 4.0
$\bar{f}_{SG}$ (%)	97.5 $\pm$ 0.3	96.3 $\pm$ 0.5	/	95.5 $\pm$ 0.9	95.9 $\pm$ 0.7	94.9 $\pm$ 0.9	92.8 $\pm$ 0.9	95.0 $\pm$ 0.8	94.6 $\pm$ 1.8
$S_{\min}$	50 $\pm$ 20	610 $\pm$ 30	/	30 $\pm$ 20	80 $\pm$ 110	110 $\pm$ 180	60 $\pm$ 30	70 $\pm$ 40	240 $\pm$ 170
$S_{\max}$	880 $\pm$ 330	1970 $\pm$ 350	/	260 $\pm$ 170	270 $\pm$ 350	360 $\pm$ 540	600 $\pm$ 270	730 $\pm$ 180	920 $\pm$ 340
$\bar{S}$	140 $\pm$ 30	930 $\pm$ 20	/	80 $\pm$ 50	120 $\pm$ 170	180 $\pm$ 280	150 $\pm$ 70	210 $\pm$ 60	430 $\pm$ 260
$\bar{S}$	71 $\pm$ 1	750 $\pm$ 23	/	42 $\pm$ 27	100 $\pm$ 140	140 $\pm$ 220	87 $\pm$ 52	122 $\pm$ 58	330 $\pm$ 240
$\bar{L}$ (μm)	270 $\pm$ 20	520 $\pm$ 120	/	320 $\pm$ 90	360 $\pm$ 200	370 $\pm$ 220	380 $\pm$ 70	420 $\pm$ 80	570 $\pm$ 100
$\bar{L}$	5.3 $\pm$ 0.2	8.4 $\pm$ 1.1	/	5.3 $\pm$ 1.1	5.5 $\pm$ 2.8	5.9 $\pm$ 3.1	6.5 $\pm$ 1.1	7.1 $\pm$ 0.9	9.0 $\pm$ 1.7
$\bar{R}$ (μm)	370 $\pm$ 30	740 $\pm$ 160	/	450 $\pm$ 130	510 $\pm$ 280	520 $\pm$ 320	520 $\pm$ 100	590 $\pm$ 110	800 $\pm$ 150
$\bar{R}$	6.5 $\pm$ 0.4	10.8 $\pm$ 1.5	/	6.5 $\pm$ 1.5	6.8 $\pm$ 3.7	7.4 $\pm$ 4.4	8.2 $\pm$ 1.5	9.0 $\pm$ 1.2	11.6 $\pm$ 2.4
$\bar{D}$ (μm)	700 $\pm$ 50	1400 $\pm$ 320	/	840 $\pm$ 250	950 $\pm$ 530	970 $\pm$ 610	990 $\pm$ 180	1110 $\pm$ 200	1520 $\pm$ 280
$\bar{D}$	12.3 $\pm$ 0.7	20.5 $\pm$ 2.9	/	12.4 $\pm$ 3.0	12.7 $\pm$ 7.1	14.0 $\pm$ 8.5	15.6 $\pm$ 3.0	17.2 $\pm$ 2.2	22.0 $\pm$ 4.3
<i>Network connectivity parameters</i>									
$\bar{C}$ (10^{-3})	26 $\pm$ 08	38 $\pm$ 13	49 $\pm$ 12	38 $\pm$ 14	44 $\pm$ 15	37 $\pm$ 04	79 $\pm$ 22	54 $\pm$ 10	37 $\pm$ 14
$\bar{E}_{\text{loc}}$ (10^{-3})	13 $\pm$ 5	23 $\pm$ 9	/	19 $\pm$ 8	23 $\pm$ 8	19 $\pm$ 2	42 $\pm$ 13	28 $\pm$ 5	21 $\pm$ 9
$\bar{E}_{\text{glob}}$ (10^{-3})	359 $\pm$ 5	297 $\pm$ 29	/	346 $\pm$ 26	356 $\pm$ 59	338 $\pm$ 55	324 $\pm$ 23	314 $\pm$ 15	276 $\pm$ 33

Abbreviations: Node parameters: $\bar{\rho}_B$ = mean branching point density, $\bar{\rho}_E$ = mean edge density, $\bar{f}_E$ = mean fraction of end nodes of all nodes, $\bar{k}$ = mean node degree, $k_{\max}$ = maximum node degree, *Graph size parameters:* $\bar{\rho}_G$ = graph density (total number of graphs per cubic millimeter), $\bar{\rho}_{LG}$ = large graph density (graphs with at least 10 edges), $\bar{f}_{SG}$ = fraction of small graphs (with less than 10 edges), $S_{\min} / S_{\max} / \bar{S} / \tilde{S}$ = minimum/maximum/mean/median graph size (of all large graphs), $\bar{L}$ = characteristic path length, $\bar{R}$ = network radius, $\bar{D}$ = network diameter, *Network connectivity parameters:* $\bar{C}$ = clustering coefficient of a node, $\bar{E}_{loc}$ = local efficiency of a node, $\bar{E}_{glob}$ = global efficiency of the network.

*Data from [164].

**Dimensionless parameters in this group are calculated by treating a graph as unweighted (distance between two connected nodes is equal to one), while quantities with a dimension refer to a weighted graph (distance between two connected nodes is equal to the physical branch length between them).

Note: The notches of the boxplots indicate the 95 % CI for the medians. Besides that, the whiskers show the 95 % CI for covering all data, i.e., the 2σ -interval for a normally distributed variable.

Node parameters

The *density of branching nodes* $\bar{\rho}_B$ describes the number of nodes per volume with a node degree of at least three. The values of $\bar{\rho}_B$ in Table 3.10 were significantly higher for the rat brain compared to the tumors. Hence, Figure 3.15 a) additionally shows that the whiskers, i.e., the 95 % CI, of rat brains and tumors do not overlap. In Table 3.10, the AT1 tumors showed a twofold lower $\bar{\rho}_B$ compared to the rat brains, UT-SCC-14 tumors showed at least twofold lower $\bar{\rho}_B$, and SAS tumors even showed at least six-fold lower $\bar{\rho}_B$. Compared to the control group of the UT-SCC-14 tumors, $\bar{\rho}_B$ increased by 15 % (conventional fractionation) and 33 % (hypofractionation). In contrast, for the SAS tumors $\bar{\rho}_B$ decreased by 8 % for the conventional fractionation and increased by 22 % for the hypofractionation. However, Figure 3.15 a) shows only a small increase of the boxplot medians of the UT-SCC-14 tumors with increasing fractionated dose and even a decrease for the SAS tumors. Thus, outliers could have been responsible for the increases in the table mean values that were not reflected in the boxplot medians.

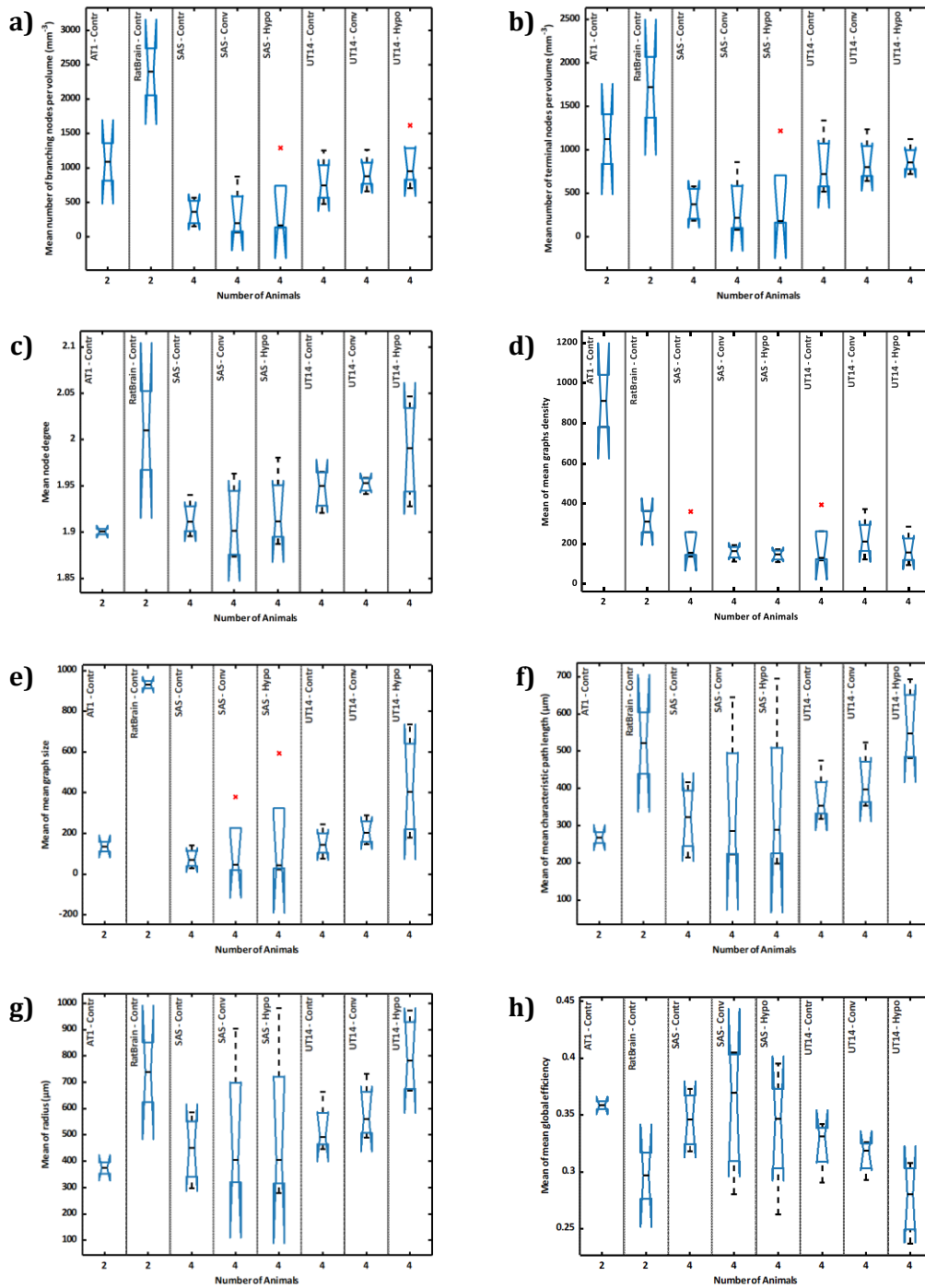


Figure 3.15. Network parameters of the largest vessel structures that have at least 10 connected edges (branches). The animals were pooled for the different tissue types and fractionation schemes. a) Density of branching nodes (node degree ≥ 3), b) density of end nodes (node degree = 1), c) mean node degree per subvolume, d) mean density of graphs per subvolume, e) mean graph size of all large graphs with at least 10 edges, f) mean characteristic path lengths averaged per graph, g) radius of each graph, g) global efficiency per graph. *Note:* The notches of the boxplots indicate the 95 % CI for the medians. Besides that, the whiskers show the 95 % CI for covering all data, i.e., the 2σ -interval for a normally distributed variable.

The *density of end nodes* $\bar{\rho}_E$ denotes the number of nodes with a node degree of one divided by the volume. $\bar{\rho}_E$ shows similar trends as $\bar{\rho}_B$, e.g., with significantly larger values for the rat brains compared to the tumors in Figure 3.15 b). Here, the rat brains' IQR (and the whisker CI hidden behind the box of the boxplot) overlaps slightly with the AT1 tumor but otherwise allows a clear separation from the other tumors at a significance level of 5 %. Deviations of the tumor $\bar{\rho}_E$ from the brain $\bar{\rho}_E$ are smaller than the deviations for the $\bar{\rho}_B$: The AT1 tumors exhibit 35 % reduced values compared to the rat brain, the UT-SCC-14 at least 48 % reduced values, and the SAS tumors at least 75 % reduced values. For the UT-SCC-14 tumors, $\bar{\rho}_E$ increases up to 9 % for the irradiated compared to the non-irradiated samples. For the SAS tumors, an increase of up to 13 % is visible for the hypofractionated irradiation, whereas the conventional irradiation deviates only slightly from the control. Figure 3.15 b) indicates a decrease in $\bar{\rho}_E$ with increasing dose for the SAS tumors, while a slight increase is observed for the UT-SCC-14 tumors. Similar trends were also found for $\bar{\rho}_B$ (Figure 3.15 a)). However, it should be noted that the partitioning into subvolumes truncates some of the vessels at the edge of the volume and these truncated vessels are also counted as artificial terminal nodes.

The *fraction of end nodes* $\bar{f}_E$ represents the fraction of terminal nodes relative to the total number of nodes. The previously mentioned truncation of vessels at the border edges also affects $\bar{f}_E$, which was at least (46.4 ± 2.3) % for all samples. In this regard, the rat brain has the smallest $\bar{f}_E$, followed by at least 5 % higher values for the UT-SCC-14 tumors, 16 % higher values for the AT1 tumors, and at least 16 % higher values for the SAS tumors.

The *mean node degree* $\bar{k}$ of all samples varies around the value of two. Thus, a large proportion of vessels exhibits only a small number of branches. The rat brain shows the highest $\bar{k}$, which are 1 % higher than the values of the UT-SCC-14 tumors, 4 % higher than the values of the SAS tumors, and 5 % higher than the values of the AT1 tumors (see also Figure 3.15 c)). In contrast, the *maximum node degree* $k_{\max}$ exhibits wider distributed values, with the brain showing the largest values. These high-degree nodes are termed *hubs* and usually play a central role in a network. The UT-SCC-14 tumor shows at least 2% lower $k_{\max}$ compared to the rat brain. In contrast, SAS and AT1 tumors deviate more from the rat brain with at least 13 %, and 14 % smaller $k_{\max}$, respectively.

Graph size parameters

The *graph density* $\bar{\rho}_G$ represents the total number of graphs per volume. $\bar{\rho}_G$ was by a factor of three higher in the AT1 tumors as compared to the rat brain (see also Figure 3.15 d)). In contrast, the UT-SCC-14 tumors exhibited a reduced number by at least 26 % compared to the rat brain and the SAS tumors by at least 36 %.

A similar trend was visible for the *density of large graphs* $\bar{\rho}_{LG}$. The name “large graphs” refers to the subset of graphs that contain at least 10 edges, i.e., connected branches. The AT1 tumors exhibited 35 % higher values compared to the rat brains, while the UT-SCC-14 tumors showed similar values as the rat brains. In contrast, the SAS tumors exhibited lower values by at least 40%.

Graphs with fewer than 10 edges were classified here as “small graphs”. For all pooled groups, the corresponding *fraction of small graphs* $\bar{f}_{SG}$ accounts for more than 93 % of the total number of graphs. Thus, a large fraction of the graphs, i.e., individual vessels, has only a few branches. Possible sources of error that could contribute to this high number are partial volume effects, but also insufficient staining of the vessel over the entire length so that the individual parts of the vessel network are interrupted. These short graphs with less than 10 edges were therefore filtered out for the following evaluations to analyze mainly the largest vascular networks.

The *size S of each graph* describes the number of edges in a graph and is represented by four parameters (minimum, maximum, mean, and median) in Table 3.10. The parameter S exhibits significantly higher values for the rat brain compared to the tumors.

The *minimum graph size* S_{min} was smaller by a factor of three for the UT-SCC-14 tumors than for rat brains, by a factor of five for the SAS tumors, and by a factor of 12 for the AT1 tumors. Besides that, the minimum graph size increased with increasing fractional dose by up to a factor of four for both mouse tumor xenografts. However, for the SAS tumors this was mainly caused by outliers since the SAS boxplot medians are almost constant (data not shown). In contrast, the medians of the UT-SCC-14 boxplots also increased similarly to the tabulated means (data not shown).

The *maximum graph size* S_{max} showed less pronounced deviations from the rat brain samples than S_{min} : The values for the UT-SCC-14 tumors were 53 % lower, 55 % lower for the AT1 tumors, and at least 82 % lower for the SAS tumors. In a similar manner to S_{min} , S_{max} increased with increasing fractional dose, but only slightly by up to 4 % and 38 % for the SAS tumors and 22 % and 53 % for UT-SCC-14 tumors, respectively.

For the *mean* ($\bar{S}$) and *median* ($\tilde{S}$) graph sizes, similar trends emerged based on the rat brain values: The AT1 tumors showed 7- and 11-fold lower values, respectively. The UT-SCC-14 tumors exhibited at least two-fold lower values and SAS shows at least five-fold smaller values for both quantities. The UT-SCC-14 tumors exhibited an increase in $\bar{S}$ and $\tilde{S}$ with increasing fractional dose from 40 % to 187 % and 40 % to 279 %, respectively. The increase for the SAS tumors was of the same magnitude with 50 % to 125 % and 138 % to 233 %, respectively. See also Figure 3.15 e) that additionally demonstrates the rise in IQR with increasing fractional dose.

The *characteristic path length* $\bar{L}$ of a graph refers to the lengths of the shortest paths between all node pairs in the graph, which are averaged over the entirety of the pairs. A large value of $\bar{L}$ usually indicates a less efficient transport of e.g., oxygen, nutrients, and metabolites, through the network. Table 3.10 shows that the tumors generally had shorter *dimensional* $\bar{L}$ than the rat brain: The lengths measured for the AT1 tumors were 48 % less, and those measured for the SAS tumors were 29 % smaller. Besides that, the SAS tumors showed an increase of $\bar{L}$ by up to 16 % with increasing fractional doses. For the UT-SCC-14 tumors, the increase with dose was even more pronounced than for SAS tumors, with an increase of up to 50 %. Thereby, the UT-SCC-14 values deviate between -27 to $+10$ % from the rat brain values, which can also be seen in Figure 3.15 f). The dimensionless $\bar{L}$ showed the same tendencies as dimensional $\bar{L}$.

The *radius* $\bar{R}$ of a network indicates the minimum of the shortest path lengths between all node pairs, while the *network diameter* $\bar{D}$ indicates the maximum. From Table 3.10, the following results can be derived regarding the dimensional parameters: Both, $\bar{R}$ and $\bar{D}$ exhibited similar trends, with values for the SAS tumors being almost constant with increasing dose. In contrast, for the UT-SCC-14 tumors an increase with increasing dose was observed, even reaching values close to those of the rat brains for the hypofractionated schedules (see also Figure 3.15 g)). Compared with the rat brains, the radii of the AT1 tumors were 50 % shorter, and the radii of the SAS were at least 30% smaller. In contrast, the UT-SCC-14 radii deviated by -30 % to $+8$ % from the rat brains.

The deviation between the unirradiated and irradiated UT-SCC-14 groups was pronounced only for the hypofractionated regimen, with a 54 % increase in $\bar{R}$. This difference was less pronounced on the SAS groups, which showed 16 % higher values for the hypofractionated group compared with the control group and almost constant values when comparing both irradiated groups.

Network connectivity parameters

The *clustering coefficient* $\bar{C}$ of a node describes the number of actual connections between its direct neighbors divided by the maximum possible number of connections. For the tissues studied in this work, low levels of clustering were generally observed, which were in the order of one-thousandth. Most of the tumor types had similar values compared with the rat brains, except for the AT1 tumors with 32 % smaller $\bar{C}$. The values for the different fractionations varied more in the UT-SCC-14 tumors compared to the SAS tumors: A deviation of up to 53 % from the control group was observed for the UT-SCC-14 tumors, whereas a maximum deviation of 16 % was found for the SAS tumors.

As an alternative to the clustering coefficient, the *local efficiency* $\bar{E}_{\text{loc}}$ of a node characterizes how well information is exchanged by its neighbors when it is removed from the network. Similar values were found for the rat brains and mouse xenografts, while AT1 tumors exhibited up to 43 % lower values than the rat brains. Again, the SAS tumors varied less than the UT-SCC-14 tumors for different fractionation schemes: The treated SAS tumors had up to 21 % lower $\bar{E}_{\text{loc}}$ compared to the control group, while the UT-SCC-14 tumors had up to 50 % lower values.

The *global efficiency* $\bar{E}_{\text{glob}}$ measures the efficiency of the entire network when all nodes in the network exchange information packets with each other. The tumors exhibited mostly higher $\bar{E}_{\text{glob}}$ than the rat brain: 21 % higher values were observed for the AT1 tumors and at least 14 % higher values for the SAS tumors. The UT-SCC-14 tumors, on the other hand, showed deviations from the brain of -8 to $+6\%$. Besides that, the UT-SCC-14 exhibited a clear trend towards lower efficiency with increasing dose (see also Figure 3.15 f)).

Branching properties of the vascular network nodes

As introduced in Section 2.3.4, real-world graph networks often exhibit so-called small-world characteristics classifying them as scale-free networks. Scale-free networks can be described by the following power-law relationship of the probability P as a function of the node degree k :

$$P(k) = c_0 \cdot k^{-\gamma}, \quad (3.7)$$

with c_0 being a scaling factor and γ being a decay parameter. The power-law behavior can be seen in Figure 3.16, which shows the relative frequencies of the node degrees and the corresponding linear fits in a double-log scale. Based on Equation (3.7), the linear regression on the double-logarithmic scale displayed in Figure 3.16 can be expressed as

$$\log(P(k)) = \underbrace{\log(c_0)}_a \underbrace{-\gamma}_{b} \cdot \log(k). \quad (3.8)$$

using two the fit parameters, the intercept $a = \log(c_0)$ and the slope $b = -\gamma$.

The regression parameters for the linear fits in Figure 3.16 for node degrees in the range $[3, 13]$ are listed in Table 3.11. The overall quality of the fits was very good with R_{adj}^2 values of at least 0.96. Consequently, the hypothesis of a scale-free network is a reasonable one.

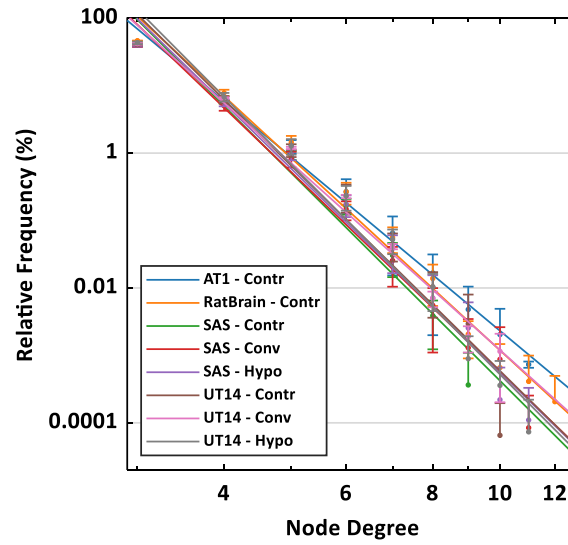


Figure 3.16. Relative frequency distributions of the node degrees on a double-logarithmic scale. The animals were pooled for the different tissue types and fractionation schemes. The relative frequencies were calculated for each animal per group and then the mean $\pm$ standard deviation of all animals per group was calculated and plotted. The linear regression on the double log scale was performed for node degrees in the range [3, 13] using a least-squares method. *Abbreviations:* Contr = control, Conv = conventional, Hypo = hypofractionated, UT14 = UT-SCC-14

Table 3.11. Linear regression parameters of Equation (3.8). The regression parameters are given as values $\pm$ standard error.

Animal	Tissue type	Fractionat.	a (Intercept)	γ (-Slope)	R_{adj}^2
Rat	AT1 tumor	Untreated	3.92 ± 0.23	8.6 ± 0.3	0.99
	Brain	Untreated	4.54 ± 0.33	9.5 ± 0.4	0.99
Mouse	Brain*	Untreated	/	$8.7 \pm 1.2^{**}$	/
		Control	4.86 ± 0.68	10.2 ± 0.9	0.96
		Convent.	4.60 ± 0.38	9.9 ± 0.5	0.98
	SAS tumor	Hypofract.	4.77 ± 0.47	10.0 ± 0.6	0.97
		Control	4.88 ± 0.84	10.1 ± 1.1	0.93
		Convent.	4.29 ± 0.35	9.2 ± 0.4	0.98
		Hypofract.	5.07 ± 0.57	10.3 ± 0.7	0.97

Abbreviations: R_{adj}^2 = R-squared, adjusted for the number of coefficients.

*Data from [164].

**The standard deviation of six samples was used here.

Many real-world networks exhibit the aforementioned scale-free behavior [164] with the decay parameter γ typically ranging between two and three [165]–[167]. Prominent examples for these real-world networks [164] include the human brain [152], metabolic [168] and protein networks [169]. In contrast, the analyzed networks showed a stronger decay with more than two-fold higher γ -values. The values for γ of the tumors were comparable to that of the rat brains: The AT1 tumors showed 10 % lower and the SAS tumors at least 4 % higher values, whereas UT-SCC-14 groups diverged from the rat brains between -3% and $+8\%$.

3.3. Simulated oxygen distributions based on experimental vascular architectures

The oxygen diffusion simulations were performed with the TOM as described in Section 2.3.5. The resulting 3D oxygen distributions for the experimental vascular architectures and 2D oxygen distributions for the simplified synthetic architectures are exemplified for AT1 tumor and rat brain in Figure 3.17. Equation (2.36) was used to calculate the number of capillaries for each synthetic 2D grid from the vascular fraction of the matching experimental subvolume. A uniform capillary radius of five micrometers was chosen for all synthetic grids, which agrees well with the measured ones (Table 3.8).

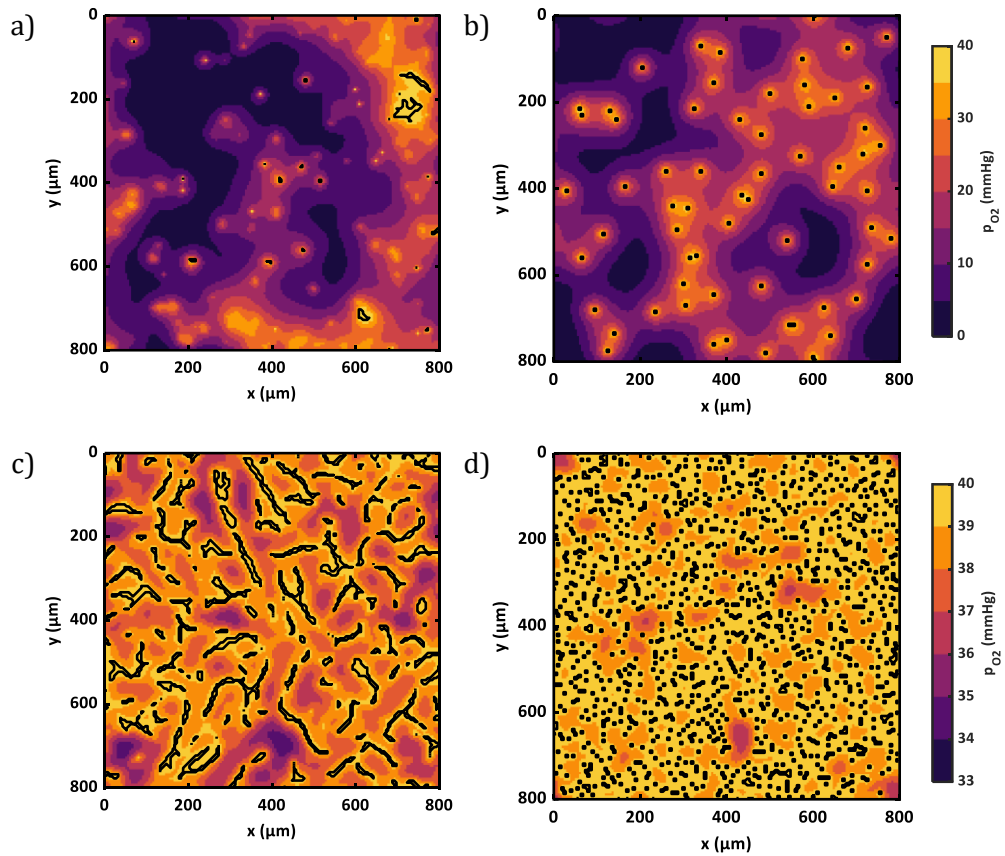


Figure 3.17. Spatial oxygen distributions for 3D experimental and corresponding 2D synthetic capillary grids with the capillary borders drawn in black. The color bars on the right belong to the images in the same row. Panel a) shows a cross-section of a 3D sub-volume of an AT1 tumor and b) the corresponding 2D synthetic grid (with the same vascular fraction of 0.77 % and a uniform capillary radius of 5 μm). Panel c) depicts a 3D subvolume of a rat brain and d) the matching synthetic grid, with each having a VF of 15 %. The color palettes indicate much better oxygenation of the rat brain grids in c) and d) as compared to the AT1 tumor grids in a) and b). In addition, the synthetic capillary grids on the right show a higher mean oxygenation level as compared to the experimental samples on the left.

3.3.1. Comparison of the 2D and 3D oxygen distributions

Table 3.12 provides an overview of the parameters for the simulated spatial oxygen distributions and the quantities describing the oxygen histograms which were calculated from the oxygen distributions.

Table 3.12. Parameters describing the spatial oxygen distributions (Figure 3.17) or their resulting oxygen histogram parameters (Figure 3.18). The data are presented as mean $\pm$ standard deviation of each quantity per group. Grouping refers to all animals of the selected tissue type treated with the same fractionation schedule. For the oxygen distribution parameters, each quantity was calculated for 10^5 tissue voxels ($\overline{\text{TVD}}$), each vessel ($\overline{\text{IVD}}$), or all hypoxic voxels, ($\overline{d}_{\text{hyp}}$) and then averaged for each subvolume. Then, the values were averaged for each animal, and the group average was calculated, as tabulated here.

		Oxygen distribution parameters			Histogram parameters	
Fractionat.	Grid	$\overline{\text{TVD}}$ (μm)	$\overline{\text{IVD}}$ (μm)	$\overline{d}_{\text{hyp}}$ (μm)	$\overline{p}_{\text{O}_2}$ (mmHg)	$\overline{\text{hf}}$ (%)
<i>AT1 tumor (rat)</i>						
Untreated	Exp.	52.4 $\pm$ 9.9	50.7 $\pm$ 1.7	63.7 $\pm$ 0.7	15.8 $\pm$ 5.2	33.0 $\pm$ 14.9
	Syn.	51.5 $\pm$ 15.8	52.4 $\pm$ 16.5	76.3 $\pm$ 4.6	17.1 $\pm$ 6.9	27.7 $\pm$ 14.6
<i>Brain (rat)</i>						
Untreated	Exp.	37.4 $\pm$ 18.9	84.9 $\pm$ 8.2	81.4 $\pm$ 17.0	31.6 $\pm$ 6.5	7.6 $\pm$ 10.6
	Syn.	17.3 $\pm$ 11.1	19.1 $\pm$ 9.7	97.5*	35.7 $\pm$ 4.0	3.1 $\pm$ 4.4
<i>SAS tumor (mouse)</i>						
Control	Exp.	99.3 $\pm$ 39.9	96.3 $\pm$ 17.6	93.4 $\pm$ 30.8	8.1 $\pm$ 3.6	58.7 $\pm$ 20.0
	Syn.	53.2 $\pm$ 35.2	56.9 $\pm$ 41.2	87.4 $\pm$ 18.3	20.9 $\pm$ 9.2	22.4 $\pm$ 26.6
Convent.	Exp.	89.6 $\pm$ 25.0	96.7 $\pm$ 13.4	87.8 $\pm$ 14.7	9.7 $\pm$ 5.7	54.3 $\pm$ 22.7
	Syn.	44.3 $\pm$ 15.7	45.4 $\pm$ 15.2	79.1 $\pm$ 1.9	21.6 $\pm$ 7.8	21.0 $\pm$ 12.2
Hypofract.	Exp.	91.7 $\pm$ 39.3	97.7 $\pm$ 22.1	90.0 $\pm$ 24.5	10.0 $\pm$ 7.7	54.2 $\pm$ 28.3
	Syn.	38.5 $\pm$ 16.6	40.8 $\pm$ 17.4	73.9 $\pm$ 1.5	22.7 $\pm$ 9.7	16.0 $\pm$ 12.4
<i>UT-SCC-14 tumor (mouse)</i>						
Control	Exp..	84.0 $\pm$ 33.5	81.2 $\pm$ 16.6	92.2 $\pm$ 18.9	13.6 $\pm$ 7.1	41.9 $\pm$ 23.9
	Syn.	29.8 $\pm$ 8.2	30.4 $\pm$ 8.4	75.4 $\pm$ 3.2	28.4 $\pm$ 4.8	8.2 $\pm$ 7.6
Convent.	Exp.	72.8 $\pm$ 11.3	80.9 $\pm$ 12.9	80.4 $\pm$ 4.9	13.7 $\pm$ 3.3	37.7 $\pm$ 11.2
	Syn.	29.8 $\pm$ 10.1	30.5 $\pm$ 9.4	78.5 $\pm$ 6.1	29.4 $\pm$ 4.3	6.2 $\pm$ 7.2
Hypofract.	Exp.	63.9 $\pm$ 9.8	86.6 $\pm$ 14.0	75.6 $\pm$ 6.6	16.2 $\pm$ 2.5	27.7 $\pm$ 8.6
	Syn.	20.5 $\pm$ 2.3	21.7 $\pm$ 2.1	73.1 $\pm$ 2.2	33.7 $\pm$ 1.3	0.9 $\pm$ 1.0

Abbreviations: Grid = type of vascular grid for the TOM simulations, Exp. = experimental, Syn. = synthetic, $\overline{\text{TVD}}$ = minimum tissue-to-vessel distance, $\overline{\text{IVD}}$ = minimum inter-vessel distance, $\overline{p}_{\text{O}_2}$ = mean p_{O_2} of the oxygen histograms, $\overline{\text{hf}}$ = hypoxic fraction ($p_{\text{O}_2} < 5$ mmHg), $\overline{d}_{\text{hyp}}$ = hypoxic distance

*No standard deviation could be displayed due to missing data for the second of the two animals.

The tissue-to-vessel distance $\overline{\text{TVD}}$, i.e., the distance of 10^5 randomly selected voxels to the nearest tissue vessel, becomes larger the smaller the vascular fraction of the tissue is. The obtained VF for this comparison can be found in Table 3.8. In addition, the $\overline{\text{TVD}}$ is an indicator of the distribution of the capillaries, i.e., larger values for the same VF would indicate a clustering of the vessels. As can be seen in Table 3.12, the $\overline{\text{TVD}}$ for the experimental simulation grids are the lowest for the rat brains, AT1 tumors exhibit 41 % higher values, followed by UT-SCC-14 tumors with 71 % larger values and the SAS with 2.4 times higher values. Thus, although the SAS and UT-SCC-14 tumors had comparable VF (Table 3.8), they showed indications that their vascular distribution is different.

This result is supported by the inter-vessel distances $\overline{\text{IVD}}$, which follow similar trends. The high value for the rat brains is likely an outlier that is not supported by its VF. The hypoxic distances $\overline{d}_{\text{hyp}}$, i.e., the distances from a pimonidazole-hypoxic tissue voxel ($p_{\text{O}_2} < 10$ mmHg) to the next vessel, were comparable for all samples, except for the slightly reduced values for the AT1 tumors. Thus, they showed no correlation with the VF.

Furthermore, the hypoxic fraction $\overline{\text{hf}}$ of the experimental oxygen histograms followed the same trends as the vessel distance parameters $\overline{\text{TVD}}$ and $\overline{\text{IVD}}$, since they all decrease with increasing vascular fraction. The mean oxygen tension $\overline{p}_{\text{O}_2}$ showed an inverse correlation to the $\overline{\text{hf}}$ and increased with increasing vascular fraction.

The comparison of the experimental with synthetic capillary grids revealed the best agreement for the AT1 tumors. This could mean that the simplified 2D linear parallel vessel architecture is a good approximation at least for this type of tumor. The other samples showed larger deviations of the parameters: Since the TVD and IVD were reduced by 50 % for the synthetic grids compared to the experimental ones, this implies that the experimental tumors have a higher absolute number of vessels distributed in the volume. The number of vessels in the synthetic grid was calculated from the VF of the corresponding experimental grid in Equation (2.36) and thus ignored the radius distribution of the measured vascular architectures.

The reduction of $\overline{\text{TVD}}$ and $\overline{\text{hf}}$ with increasing fractional dose was more pronounced for the UT-SCC-14 tumors than for the SAS tumors and can be linked to the increase in VF for both tumor lines.

3.3.2. Comparison of the oxygen histograms

The comparison of oxygen histograms of the experimental and synthetic capillary architectures, depicted in Figure 3.18, yielded similar results as the previously discussed spatial parameters, i.e., the least deviations for the AT1 tumors having three-fold lower $\overline{\text{EMD}}$ compared to the rat brains. Larger deviations were found for the rat brain ($\overline{\text{EMD}}$ of 11.0 %), the SAS (three-fold higher $\overline{\text{EMD}}$) and UT-SCC-14 tumors (four-fold higher $\overline{\text{EMD}}$).

Table 3.13. Quantitative comparison of the oxygen histograms of the experimental with synthetic capillary simulations, depicted in Figure 3.18.

Tissue type	Fractionation	$\overline{\text{DER}}$ at 1 % SF	$\overline{\text{EMD}}(\%)$	$\overline{\text{MAD}}(\%)$	$\overline{\text{MaxAD}}(\%)$
<i>Rat</i>					
AT1 tumor	Untreated	1.04	3.4	0.7	4.9
Brain	Untreated	1.08	11.0	4.0	32.1
<i>Mouse</i>					
SAS tumor	Control	1.19	34.0	5.7	30.4
	Convent.	1.20	31.5	4.6	29.8
	Hypofract.	1.26	33.9	5.2	33.7
UT-SCC-14 tumor	Control	1.33	39.3	5.8	28.7
	Convent.	1.33	41.8	6.6	24.6
	Hypofract	1.37	46.4	7.7	34.5

Abbreviations: DER = dose enhancement ratio, EMD = earth mover's distance, MAD = mean absolute deviation, MaxAD = maximum absolute deviation

Figure 3.18 shows in addition that the most pronounced deviations occur for the first (severe hypoxic) and the last histogram bins (high oxygenation). Besides that, a right-shift of the synthetic curves with higher fractional doses is visible, which is more pronounced for the UT-SCC-14 tumors compared to the SAS tumors and more evident for the synthetic compared to the experimental simulation grids.

Thus, there seem to be inherent differences in the experimental capillary grids as compared to their corresponding synthetic ones simulated with the same VF matched for each subvolume, which limits a better agreement. AT1 tumors were an exception with a good agreement. Reasons for these deviations are most likely a generally different capillary distribution in the experimental samples, which deviated from the assumed randomly distributed synthetic capillaries. Therefore, the experimental samples exhibited a lower fraction of highly oxygenated regions (Figure 3.18) and consequently increased hypoxic fractions.

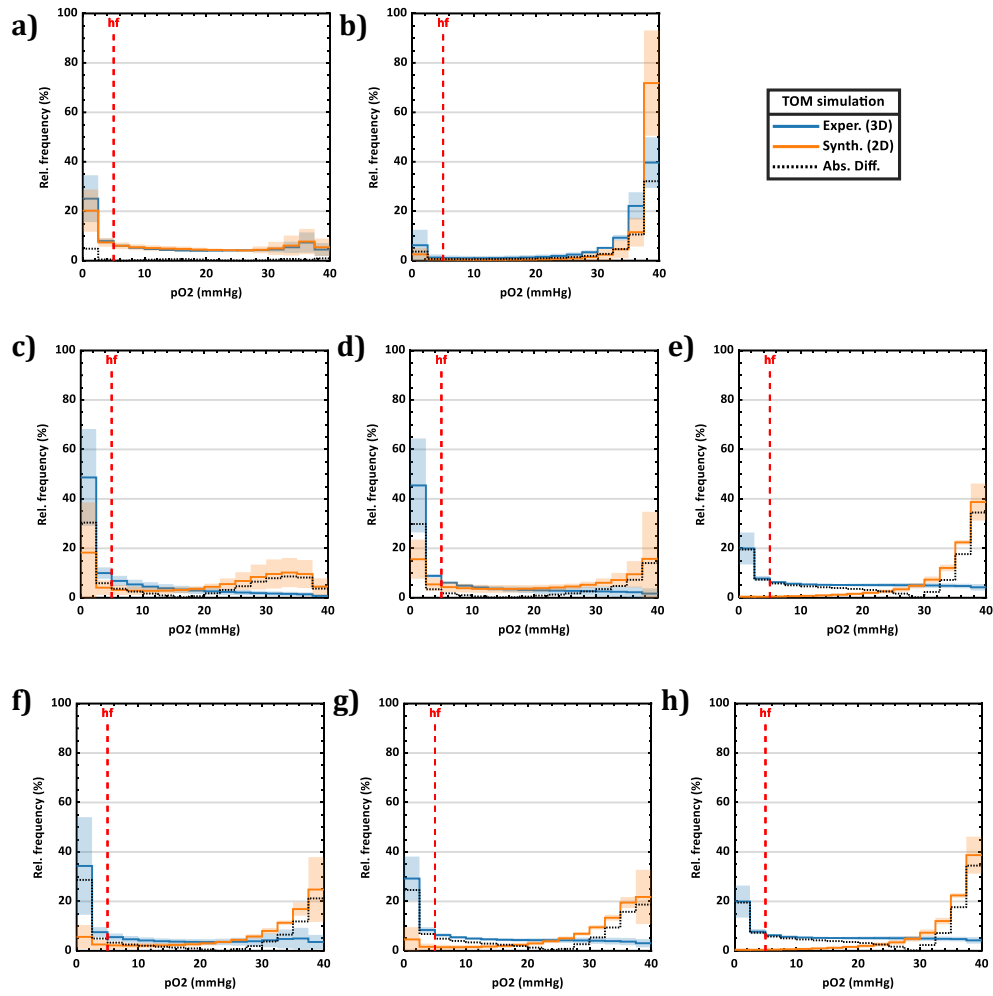


Figure 3.18. Comparison of the Oxygen histograms for 3D experimental and 2D synthetic capillary distributions. The oxygen histograms show the means $\pm$ standard deviations per group. Grouping refers to all animals of the selected tissue type treated with the same fractionation schedule. The histograms of the experimental samples were calculated for each subvolume, followed by an averaging for each animal. 20 synthetic capillary grids were generated for each subvolume with the same vascular fraction and averaged for the subvolume. For both data sets, the group average and standard deviation were then calculated, as illustrated here. The panes depict a) AT1 tumors, b) Rat brains c), d), e) the SAS tumor groups “control”, “normal” and “hypofractionation”, and f), g), h) the UT-SCC-14 tumor groups “control”, “normal” and “hypofractionation”.

4. Discussion

The discussion closely follows the structure of the results section. First, the validation of the numerical accuracy of the tumor oxygenation model (TOM) is discussed. Then, the comprehensive empirical characterization of the vascular architecture of the experimental tumors and normal tissues is performed. The last part covers the simulation of the oxygen distribution in the experimental tumors with the updated version of the TOM, which was enabled by the two previous evaluations.

4.1. Validation of the numerical accuracy of the diffusion-reaction equation solutions

Section 3.1 describes the mathematical validation of the numerical results of the TOM, which was developed in-house by I. Espinoza to simulate the oxygen diffusion from capillaries to the tissue [170]. The framework of the TOM was described in detail in Espinoza et al. (2013) [27] and based on this simulation study, similar calculations were performed e.g. by Warren and Partridge (2016) [94] and Paredes-Cisneros (2018) [157] using different numerical methods. The authors reported distinctly different oxygen distributions as shown in Figure 3.1 of Section 3.1.1.

To analyze these variations in the oxygen histograms, a numerical validation of the TOM was performed as part of this thesis. As a first step in the validation pipeline, the diffusion profile of a single capillary was compared for the numerical solution with the TOM and the analytical solution by Grimes et al. (2014) [84]. The diffusion profiles of the TOM in Figure 3.4 of Section 3.1.1 revealed a severe underestimation of the oxygen partial pressures in the published TOM version of 2013. Only a combination of several algorithm changes led to a good agreement with the analytical solution and a good agreement between the originally implemented numerical particle strength exchange method (PSEM) and the finite difference method (FDM), which was additionally implemented in this thesis:

- (i) Adapting the TOM algorithm to be voxel size-independent using the Courant-Friedrichs-Lewy (CFL) condition.
- (ii) Increasing the spatial resolution of the simulation grid by reducing the voxel size to a reasonable value.
- (iii) Changing the cross-sectional shapes of the synthetic capillaries from rectangular to circular shapes.

- (iv) Introduction of a two-stage termination condition for the simulation when it reaches the equilibrium position.

These algorithm modifications are described in detail in the following four sections.

4.1.1. Adapting the TOM algorithm to be voxel size-independent

To fully achieve the voxel size independence, the Courant-Friedrichs-Lewy (CFL) condition had to be implemented in the FDM and optimized for both PSEM and FDM as described in Section 3.1.3. The CFL condition improved the runtime and stability of the simulations independently of the voxel size but it also revealed a disadvantage of the explicit numerical schemes, used in this work and by the before mentioned authors: The number of time steps scales with the square of the voxel size. Thus, reducing the voxel size to achieve a higher accuracy leads to an increased number of iterations and consequently to a higher computational effort. Due to the complex vessel geometries used in this work, only an explicit numerical solution is possible, which limits the simulations to volumes of a few cubic millimeters for the standard parameter sets used.

4.1.2. Increasing the spatial resolution of the simulation grid

In Espinoza et al. (2013) [27], a standard simulation setup for the TOM simulations included simplified linear capillaries and voxel sizes of 20 μm . For a graphical representation of the simulation grid as in Figure 3.4, one voxel is typically assigned to each grid point, with the grid points at the center of the voxels.

A schematic representation of the diffusion profile of a single capillary can be found in Figure 3.5. In this illustration, four aspects are visible that affect the accuracy in the standard simulation setup from [27]:

- (a) The diffusion starts already inside the capillary due to its point-shaped cross-section, although a constant capillary p_{O_2} of 40 mmHg is assumed for the entire capillary volume.
- (b) The entire diffusion profile is only represented by a single-digit number of grid points.
- (c) The rectangular capillary cross-section overestimates the capillary volume compared to a more realistic assumption of a circular cross-section.
- (d) The number of capillaries was calculated from the vascular fraction and was thus dependent on the voxel size of the simulation volume.

The cause for point (a) is, that in the simulation settings of TOM version 2013, only one grid point represents the capillary cross-section. This is because the simulation grid for the TOM simulations is a point grid, as shown in Figure 3.5. Thus, the lateral

spatial extension of the capillary is ignored and the lateral oxygen diffusion profile drops already between the capillary grid point and the next adjacent (tissue) grid point. Half of the distance between these two points should count as space within the capillary when the spatial extension of the capillary with a diameter of $20\ \mu\text{m}$ is considered. Thus, the oxygen partial pressure already drops within the capillary, although a constant p_{O_2} of 40 mmHg is assumed for the whole capillary volume in the TOM. Consequently, the oxygen diffusion profiles at this resolution on a (2D simulation grid) refer to point-like capillaries, despite the assumed finite volume reflected by the vascular fraction.

As a result, the maximum diffusion distance is reduced by 50 % of the voxel size, since half of the capillary voxel should be counted as part of the diffusion profile. This effect is shown as a shaded area in the oxygen profile in Figure 3.2. Besides that, Figure 3.7 shows that one would expect a lateral diffusion profile with a maximum diffusion distance of ca. $120\ \mu\text{m}$. The maximum diffusion distance is here defined as the distance, where the p_{O_2} drops to 1 % of the initial capillary p_{O_2} . Other simulation studies reported slightly higher values, such as $143\ \mu\text{m}$ [43], $145\ \mu\text{m}$ [59], and $150\ \mu\text{m}$ [170]. In contrast, experimental studies showed a wide range of distances depending on the tumor type, such as $150\ \mu\text{m}$ in [59], [80], and [171] and the range of $[107, 193]\ \mu\text{m}$ in [171].

The consequences of this effect are even more pronounced for a multi-capillary setting since the inter-capillary distances are overestimated by ignoring their finite volume and thus the oxygenation of the simulated volume is reduced. Additionally, because of the point-shaped capillary, the neighboring voxel in the tumor already receives significantly lower oxygen levels than if diffusion were to start at the edge of a capillary with a spatial extension. This neighboring voxel is classified as “tissue voxel” and represented in the resulting oxygen histograms, while the “capillary voxels” are not included in the histograms. Thus, the highest bins in the oxygen histograms are underrepresented. To overcome this problem, a smaller voxel size of micrometers or below leads to a significantly larger number of grid points, also within the capillaries, and thus assigns the capillary oxygen tension of 40 mmHg essentially to the whole capillary cross-section. As a result, the oxygen profile starts decreasing only from the geometrically defined capillary border that gets better represented at a higher spatial resolution and a more realistic capillary shape with a circular cross-section, as shown in Figure 3.7.

Regarding (b), the limited number of grid points for a voxel size of $20\ \mu\text{m}$ means, that for a diffusion profile with a maximum diffusion distance of $120\ \mu\text{m}$, only seven grid points, are used to represent the profile, as shown schematically in Figure 3.5. Such a low number of grid points is in general a too coarse approximation for a non-linear

differential equation. Consequently, numerical algorithms such as FDM and PSEM underestimate the p_{O_2} in the profile and start to approach the exact value of the curve only for higher resolutions and thus a higher number of simulation grid points.

For point (c), the shape of the capillary cross-section was analyzed in Section 3.1.3. The shape had a major impact on the space-filling of the capillary on the simulation volume and the deviations from the analytical solution. These deviations could be reduced by employing a circular capillary cross-section instead of a squared one, which is possible only for voxel sizes of five micrometers and below. As a result, the radial diffusion profile of circular capillary cross-sections had the smallest maximum absolute deviation from the analytical solution in Figure 3.7. An additional advantage of the circular capillary shape is that it is closest to the natural capillary shape, increasing the realism of the simulation.

For multi-capillary simulations as in Section 3.1.4, a space-filling capillary was beneficial as well, since the distance to the next capillary is better approximated than with punctiform capillaries. Thus, the whole oxygen distribution was right-shifted to more oxidic values, which can be seen in Figure 3.10.

To correct for (d), the TOM algorithm was modified as follows: The number of capillaries in a synthetic simulation volume is calculated with Equation (2.36) from a given vascular fraction. In the modified code version, an ideal circular capillary cross-section is assumed for the calculation of the number of capillaries. This means that the previously considered vessel extension on the voxel grid is now ignored, decoupling the calculations from the voxel size. Consequently, the code modification leads to more oxidic histograms compared to the TOM 2013 version, as depicted in Figure 3.12 of Section 3.1.5.

4.1.3. Comparison with an analytical solution of the DRE

Some deviations of the two numerical methods from the analytical solution, which were analyzed in Section 3.1.2, were found to be inherent and constant for different voxel sizes. This could be seen, e.g., when comparing the maximum diffusion distance, and should be considered when performing TOM simulations. The most likely explanation for the deviations lies in the approximations of the analytical solution: As the consumption term in Equation (A.7) of Appendix A is linked to the maximum diffusion distance where the oxygen tension drops to zero, the oxygen-dependent consumption follows a quadratic form instead of a Michaelis-Menten shape. Thus, the TOM and the analytical solution solve two different mathematical problems. This leads to a slightly larger oxygen diffusion distance for the analytical solution since its quadratic consumption term leads to a lower oxygen consumption close to the maximum diffusion distance compared to the Michaelis-Menten consumption.

Since the validation of the accuracy of the two numerical methods PSEM and FDM in this work are based on the analytical solution of Grimes et al. (2014) [84], the comparability of numerical solutions with the analytical solution influences the validation procedure. Consequently, a new analytic approach that does not include the simplification of the oxygen consumption term could further improve the comparability between the analytical and numerical solutions and thus increase the reliability of the numerical algorithms.

4.1.4. Introduction of a two-stage termination condition for the simulation

The implementation of a two-stage termination condition was necessary to estimate a meaningful number of simulation iterations until the oxygenation in the grid reaches a steady state and additionally the shape of the oxygen histograms remains stable. The condition is schematically illustrated in Figure 3.8 of Section 3.1.3. Since the number of iterations strongly depends on the simulation grid properties, such as voxel size, simulation volume, and vascular architecture, a second condition was introduced to control the stability of the oxygen histograms. The second condition is thus independent of the grid properties so that the two-stage process can be easily applied for different simulation conditions without manually adapting the number of iterations.

4.1.5. Validation of the TOM simulation framework

Both numerical algorithms, PSEM and FDM, led to comparable mathematical accuracy for individual capillaries, as depicted in Figure 3.7 c) and d). Besides that, the oxygen histograms are shown in Figure 3.11, which were generated with both algorithms for the same multi-capillary grids'. The histograms exhibited only slight deviations from each other: The FDM is slightly advantageous because of the faster algorithm runtime since only directly neighboring grid points are considered instead, as for the PSEM, all neighbors within a radius of five voxels.

Both algorithms should be ideally used in combination to estimate the achievable mathematical precision with these explicit numerical schemes and to avoid numerical inaccuracies of one method. These inaccuracies can occur, for example, with certain parameter combinations where the CFL condition is violated, or with certain vascular architectures where one of the solvers may fail to reach a steady state of the solution.

4.1.6. Summary of the validation steps

In summary, to validate the numerical accuracy of the TOM, the simulation framework was extended to be independent of the voxel size, which was validated for the voxel range $[1, 20] \mu\text{m}$. Besides that, the FDM was implemented as a second numerical method. After implementing the four algorithm modifications discussed above, a multi-capillary setting was used to validate that both numerical methods, PSEM and FDM, generate the same numerical results for the same problem.

After this validation, the optimized *TOM 2021 version* was established with a set of four parameter combinations listed in Table 3.5. The different parameter combinations (FDM or PSEM and standard or high-precision for each method) for the TOM simulations in Section 3.1.5 led to highly consistent results. Besides that, transferring the resulting oxygen histograms into simulated survival curves yielded dose enhancement ratios (DER) of 1.00 when comparing the four different parameter combinations. Thus, the different numerical methods and precisions do not influence the calculated radiation response, i.e., all settings listed in Table 3.5 result in the same survival probability and could be used equivalently in radiobiological models, e.g., the *tumor response model* (TRM) [28].

The TOM 2012 version results in a right-shift of the simulated oxygen histograms compared to the *TOM 2013* version from [27]. This right-shift is exemplified in Figure 3.12 of Section 3.1.5 and led in the example to a DER of 1.22 of the old compared to the new TOM version. Consequently, TRM simulations as in [28] and [172] using the new TOM version will be affected as well: The updates will result in higher radiation sensitivities of the simulated tumors and thus a left-shift of the dose-response curves and reduced TCD_{50} values.

4.2. Empirical characterization of the tumor vasculature

Section 3.2 provides a comprehensive characterization of tumor and normal tissue vasculature as measured by fluorescence microscopy on 2D histological sections and 3D light-sheet fluorescence microscopy (LSFM) for whole tissue samples. The analysis was performed in three steps, which are discussed in the following sections: First, the parameters that can be evaluated by both methods were compared. In the next section, the tissue types were then compared using conventional 3D vessel parameters. Subsequently, network parameters were evaluated, using a graph-theoretical approach to analyze the vascular architecture as 3D vessel networks.

4.2.1. Comparison of 2D histological and 3D LSFM vascular parameters

Good agreement of the methods for vascular fraction and mean radius

The comparison between vascular parameters measured with 2D histology and 3D LSFM in Section 3.2.1 showed good agreement between the methods regarding the vascular fraction and the vessel radii averaged per 2D slice (histology samples) or 3D subvolume (LSFM samples).

Furthermore, based on the vascular fraction, each of the subvolumes could be reliably distinguished between the rat brain samples and the tumors. However, SAS tumors in the conventionally treated group were an exception, as for them the CI whiskers in Figure 3.11 c) did not show a clear separability from the rat brains. In summary, quantitative analysis of LSFM images was shown to agree quite well with the same parameters as conventionally obtained 2D histology images.

Compared to the results of Hahn et al. (2019) [164] who used a similar image processing pipeline including LSFM imaging and segmentation with ilastik, the mean vessel radii of their mouse brain samples matched well to the rat brain samples used in this work. This is an indication that the LSFM pipeline established in this work provides fundamentally reliable results.

Differences between both methods for the median radii

However, the median radii, i.e., the median of the radii for a 2D slice (histology) or a subvolume (LSFM), exhibited major differences (see Figure 3.13). This can be attributed to the following three differences in the measurement methods:

- (i) The lateral resolution of the image acquisition.
- (ii) The method for calculating the radius.
- (iii) The total number of vessel radii measured.

Regarding (i), the lateral resolution of the fluorescence microscope for acquiring 2D images of the 10 μm thick histological sections was less than 0.44 μm [114, p. 71]. However, after correction for tissue shrinkage, the 3D images acquired with the LSFM were only measured with an in-plane resolution of 2.4 μm and a slice thickness of 5.9 μm . Besides that, in the LSFM setup, the antibody is excited to fluorescence at a larger distance from the object (up to a few millimeters) compared to the 2D method, which leads to a stronger scattering of the signal in the sample. This also leads to an artificial enlargement of vessels with increasing distance to the objective. In addition, more autofluorescence is observed in the LSFM samples due to the dimmer signal from the IHC stained vessels compared to 2D, resulting in a lower signal-to-noise ratio. Consequently, the 3D radius measurement is more error-prone.

Concerning (ii): The vessel radius of the 2D samples was calculated by fitting an ellipse to each vessel outline and using the length of the shorter semi-axis as an estimate of the radius [114, p. 74]. In contrast, the radius for the 3D samples was computed as the shortest distance from the vessel centerline (given by the vessel skeleton) to the nearest non-vessel voxel. Thus, the 2D radius accounts for the shape of the vessel, while the 3D method measures only a distance between two voxel centers and is thus less informative.

It follows from (ii) for (iii), that there is a much larger number of measurement points for each vessel in 3D, which improves the statistics. Besides that, for the 2D samples, at least two adjacent tissue sections per animal were taken close to the center of the tumor sample [114, p. 74]. This means that, on the one hand, only a small number of slices and thus individual vessel radii could be determined, which were typically in the two- to three-digit range per animal. In contrast, for the 3D datasets, half of the entire tumor could be evaluated. Hence, at least a volume of 20 mm³ of tissue per animal was available with at least 400 vessels per cubic millimeter (Table 3.9).

4.2.2. Conventional 3D vascular parameters

The analysis of the conventional 3D vascular parameters in Section 3.2.2 showed good differentiability between tumor and brain samples. In particular, the two quantities total vessel length density as a radius-independent alternative to the vascular fraction and the mean number of branches per node revealed meaningful differences (see Figure 3.14). The AT1 tumor could be distinguished from the other tumors using the parameters mean tortuosity and minimum inter-vessel distance. The SAS and UT-SCC-14 were difficult to distinguish with conventional vessel parameters due to their high intra-tumoral variabilities. Here, the total vessel length density and the mean number of branches per node were the most promising parameters for differentiation. Furthermore, no change after irradiation was detectable for the vascular parameters studied.

Branching parameters

The mean branch lengths of the rat brains in Table 3.9 were nearly a factor of three higher compared to the mouse brains from [164]. The reasons for this could in principle be fundamentally different properties of the tissue types. However, it is more likely that different staining qualities of the mouse and the much larger rat samples, which were stained with lectin in both experimental setups, influenced the results.

Density parameters

The density parameters in Table 3.9 indicated no clear trends for radiation-induced alterations of the vascular networks of SAS or UT-SCC-14 tumors. The UT-SCC-14 tumor showed twice the density of branches per volume as the SAS tumor with a comparable density of vessels per volume, indicating a larger branching of the individual vessels and thus angiogenetic activity.

Distance measures

In contrast to the 2D histology, the evaluation of the 3D vasculature provides more accurate information on the parameters tissue-to-vessel distance and inter-vessel distance. These distance measures may be used as a surrogate for possible hypoxic volumes, which are so far not directly measurable with LSM.

The rat brains and the AT1 tumors were both stained with lectin and thus only the perfused vessels were marked and included in the analysis. The SAS and UT-SCC-14 tumors, on the other hand, were stained with CD31, so that all vessels, regardless of their perfusion status, were included. Thus, a better correlation between oxygenation and vascular architecture was possible for lectin-stained samples compared to the CD31-stained samples.

The fraction of perfused vessels in the two mouse xenografts was determined, for example, in [22], [51], [114], [173] (see Table C.1 in Appendix C). The perfused fraction of unirradiated tumors was determined e.g., in [22] to be $(76.9 \pm 10.7) \%$ for the SAS tumors and $(83.5 \pm 7.6) \%$ for the UT-SCC-14 tumor. These values represent mean values $\pm$ standard deviation over 47 – 60 animals. This means that roughly 20 % of the vessels of the mouse xenografts stained with CD31 must be considered as being not perfused and thus do not contribute to the oxygen supply. Consequently, the distance measures for the SAS and UT-SCC-14 tumor lines would lead to larger values when perfusion would be considered

The tissue-to-vessel distance was twice as high for SAS and UT-SCC-14 tumor lines compared to the rat brains (Table 3.9) indicating poorer oxygenation in these tissues. Thereby, irradiation had less effect in the SAS groups, whereas a greater decrease in TVD was observed in the more radiosensitive UT-SCC-14, suggesting possible angiogenesis and thus reoxygenation.

4.2.3. Advanced graph parameters of the vascular networks

The network parameters in Table 3.10 of Section 3.2.3 exhibited good discrimination of the three tissue groups: AT1 tumors, rat brains, and mouse xenografts, i.e., the SAS

and the UT-SCC-14 tumors. Useful parameters for this separation were, e.g., the average density of large graphs, i.e., vessels that consist of at least 10 branches, in the volume or the four graph size parameters ($S_{\min}, S_{\max}, \bar{S}, \tilde{S}$).

Figure 3.15 additionally illustrates that the rat brain, as healthy normal tissue, exhibited the largest vascular connectivity and density, and thus an optimal oxygen supply to the entire organ. Besides that, the higher fraction of terminal nodes for all tumors compared to the brains could indicate dysfunctional vessels ("dead ends"), but also the formation of new vessels, i.e., angiogenesis.

Comparison to mouse data from Hahn et al. (2019)

The literature values of $\bar{f}_E$, $\bar{k}$ and $k_{\max}$ for the mouse brain data from Hahn et al. [164] were distinctively different from those of the rat brain measured in this work. This means, that differences in the tissues themselves and basic methodological differences must have prevented a better agreement: $\bar{f}_E$ was almost a factor of four smaller in the mouse brains of [164] compared to the rat brains used in this work. Besides that, the mouse brains' nodes appeared to be more branched with a 61 % higher mean node degree and a fourfold higher maximum node degree. However, the resolution of the mouse samples hardly allows the detection of such high maximum node degrees. Thus, it is more likely that the limited resolution of the LSFM makes several nodes appear as a single node with a high node degree.

The clustering coefficient of the mouse brains was 29 % higher than the coefficient of the rat brains, but the values agreed within their 1σ -ranges. Thus, both tissues have a similar number of loops, i.e., alternative connections of adjacent vessels of a vessel. These loops are not expected in healthy brain tissue, since they represent redundant capillary connections. Thus, they could be indicative of adjacent vessels that were counted as one branching vessel due to the limited LSFM resolution.

Dose-dependent changes

The dose-dependent changes were more pronounced for the more radiosensitive UT-SCC-14 tumors compared to the SAS tumors, e.g., visible in panels e) – f) of Figure 3.15. Additionally, the increased intra-tumoral heterogeneity in these panels was more pronounced in the SAS samples compared to the UT-SCC-14 tumors. For the UT-SCC-14 tumors, the increasing graph sizes and radii for higher fractional doses implied that the vascular networks were larger after irradiation compared to the unirradiated networks. The large graph sizes may be a result of angiogenesis. Additionally, since the number of viable cells in irradiated tumors is reduced by irradiation, these two factors could lead to reoxygenation of the remaining viable tissue in irradiated tumors and thus to increased availability of oxygen.

Transport efficiency of the vascular networks

At the same time, however, the increasing characteristic path lengths and decreasing global efficiencies of the network indicated that the irradiated networks had longer oxygen transport distances across the vascular network. Furthermore, a possible failure of individual nodes in irradiated networks would affect the transport efficiency more in these networks compared to the control group. Therefore, oxygen transport in irradiated networks would be less robust and the failure of hubs would limit transport more.

Branching properties of the vascular network nodes

The experimental vascular networks exhibited a power-law decay of the frequency distribution of the node degrees (see Figure 3.16 of Section 3.2.3). This behavior allowed the classification of the studied networks as scale-free networks, which usually possess a short-tailed node degree distribution and a small fraction of central nodes.

Compared to the mouse brain data of Hahn et al. [164], the rat brains investigated in this work exhibited a 9 % higher value for the decay parameter γ of the power law distribution. This increase can be attributed to two differences in the characteristics of the networks: (i) For the samples studied in this work, the mean node degree ranged from 1.96 to 2.01, which was 10 to 15 % lower compared to the mouse brain data with a $\bar{k}$ of 3.24 (see Table 3.10). Consequently, most of the nodes of the samples studied in this work had lower node degrees, and thus the proportion of hubs was also less. (ii) The maximum node degrees, i.e., node degrees of nodes classified as hubs, were 77 to 81 % lower for the samples of this work with $k_{\max}$ of 3.96 to 4.7 compared to the mouse brain samples with a $k_{\max}$ of 20.7. Possible reasons for the lower values are likely to be due to differences in the experimental workflows, especially regarding the clearing and staining processes of the samples and the image analysis pipelines. Besides that, the decay parameters of the tumors studied in this work did not show significant differences to those of the rat brains.

4.3. Simulated oxygen distributions using experimental vascular architectures

The simulated oxygen distributions and oxygenation measures in Section 3.3 revealed low comparability between 2D experimental results and 3D simulations. For example, compared to the hypoxic distances measured in the 2D slices (see Table C.2 in Appendix C), the mean hypoxic distance $\bar{d}_{\text{hyp}}$ was twice as high for the simulated 3D SAS tumors. Since the hf exhibited similar trends, the comparability of the simulated and results measured in 2D is limited. An explanation for this divergence may be that the 2D histology images, stained with pimonidazole as hypoxia-marker, contain both

acute and chronic hypoxic regions. The TOM simulations on the other hand consider only chronic (spatio-temporally constant) hypoxia. Thus, acute hypoxic regions, which are usually in spatial proximity to transiently non-functioning vessels, result in lower values for $\bar{d}_{\text{hyp}}$ as compared to chronic hypoxia.

Furthermore, it could be shown that the synthetic parallel linear architecture used in the TOM to approximate the experimental vascular architecture showed a good agreement for the AT1 tumors (see Figure 3.18). In contrast, the experimental samples of the other tumors exhibited larger hypoxic fractions and lower fractions of highly oxygenated areas as compared to the synthetic vascular grids. The randomly distributed capillaries of the synthetic vascular architecture could be a reason for this deviation.

Besides that, the shift of the synthetic grids to higher oxygen levels compared to the experimental samples can also be attributed to large variations of the capillary radii in the experimental samples around the mean radius of five micrometers. This variation is typically observed as a log-normal distribution [92], [164] with a long tail that is simplified by a single value for the synthetic capillaries.

Equation (2.36) was then used, to calculate the number of capillaries in the synthetic grid from the vascular fraction of the experimental grid. Since each of the large radius-capillaries would fill a larger volume compared to an average-sized capillary, they would increase the VF while each being only a single source of oxygen for the simulations. Consequently, several capillaries in the synthetic grid with a uniform volume and radius of five micrometers were computed to approximate one experimental capillary with a large radius. Hence, the number of oxygen sources was higher in the synthetic grid and thus the tissue oxygenation increased accordingly. Improving the simplified synthetic architectures would therefore involve sampling the experimental radius probability distribution to better approximate the number of capillaries.

4.4. Limitations of the LSFM microscopy

Simultaneous immuno-histochemical (IHC) staining of the 2D histological sections with multiple dyes allows the extraction of multiple tissue characteristics, such as hypoxia, necrosis, perfused and non-perfused vessels, in the same data set. In contrast, the LSFM method is limited in the number of already established IHC dyes. Thus, for the tumor samples used in this work, only staining of perfused vessels with the fluorescent marker lectin and non-perfused vessels with the marker CD31 could be successfully achieved. In this regard, the limited detectability of pimonidazole-stained hypoxic volumes in our LSFM setup is likely due to insufficient diffusion of the corresponding secondary antibody within the tissue.

Another limitation of the LSFM results from the masking of volumes without a fluorescent signal of the vessels, e.g., due to necrosis or insufficient diffusion of the dye. This particularly led to an exclusion of central regions in irradiated tumors, which usually did not occur in non-irradiated tumors. Thus, particularly in the hypofractionated tumors, the tumor centers exhibited no signal and were therefore excluded from the analysis.

The partitioning of the bulk sample into smaller subvolumes resulted in the truncation of vessels at the volume boundaries. The truncation artificially shortened the branch lengths and increased the number of vessel end nodes. Besides that, the incorrect assignment of the node degree two caused an increase in the number of branching nodes and thus shortened measured lengths of the individual branches.

The novelty of the LSFM method also implies that certain processes, such as clearing and IHC staining, must be established individually for each animal and organ. For example, the insufficient diffusion of the secondary antibody for pimonidazole in the present work prevented establishing this fluorescent marker as a hypoxia marker. Furthermore, the data available in the literature from 3D characterizations of tumors at the microscopic scale are very sparse, as the histological gold standard is mostly used. The more laborious 3D imaging methods are only slowly becoming widespread.

The comparison of the results of this work with those of Hahn et al. (2019) revealed large discrepancies that can be attributed to methodological differences. Therefore, standardization of LSFM workflows, especially for the clearing, staining, and vessel characterization steps, is necessary to validate the results and improve the reliability of the LSFM method.

5. Conclusion

The validation of the numerical solutions of the diffusion-reaction equation (DRE) in Section 3.1 showed that a careful selection of the simulation parameters is crucial to obtain numerically accurate results. After implementing the finite differences method (FDM) as a second numerical method, the stability and numerical accuracy of the simulated oxygen distributions were evaluated for both the FDM and particle strength exchange method (PSEM).

Two parameter sets were established for different required numerical precisions (Table 3.5) and both settings revealed consistent results for both methods (Table 3.6). In addition, the algorithm changes of this work for the updated TOM 2021 version resulted in a generally higher oxygenation of the average tissue voxel for typical vascular fractions compared to the original TOM 2013 version. This caused a right-shift of the new oxygen distributions compared to the original TOM version (Figure 3.12), which subsequently leads to an increased radiation sensitivity of tumors in tumor response model (TRM) simulations.

Consequently, by benchmarking the TOM framework and the mathematical validation, the numerical accuracy of the TOM could be improved and a general recommendation for reliable oxygen distribution simulations could be established. As there is no standardized model to solve the diffusion-response equation (DRE) with an oxygen transport simulation model such as the TOM, these recommendations could be a first step to validate the DRE algorithms used by different authors. This could be useful to increase the reliability and comparability of different studies, such as those shown in Figure 3.1.

With the new *TOM 2021* version, developed in this work, a numerically accurate oxygen distribution can now be determined for a given vascular architecture. This allows the comparison of different tumor tissues concerning their vascular architectures by using the oxygen distributions simulated with the TOM as an input for other models. One model could be, for example, the *tumor response model* (TRM) developed in-house by I. Espinoza et al. [28] that can be used to simulate the radiation response of tumors. The TOM histograms are used in the TRM for two processes: the oxygen-dependent radiation-induced cell kill and angiogenesis. Angiogenesis is implemented

such that vascular development occurs when hypoxia is present in a voxel [28]. Consequently, the TRM allows to study the oxygen-dependent radiation response of tumors during treatment and to draw conclusions about their radiosensitivity.

The comparison of the 2D and 3D microscopy images of experimental tumors with different radiosensitivities in Section 4.2.1 provided a good agreement of both methods. The comparison also highlights the advantages of each method: The 2D histology allowed simultaneous staining with multiple IHC markers and imaging with higher resolution. On the other hand, the LSM microscopy has the advantage that the 3D vascular network of an entire tumor can be analyzed without artifacts from tissue cutting, 3D reconstruction of histological sections, etc. The 3D analysis of the vascular network in this work showed for example a good separability between different tissue types using parameters such as the number of interconnected vascular branches. In addition, it indicated possible radiation-induced changes in the network, such as a reduced global transport efficiency at a higher fractionated dose.

The LSM analysis pipeline established in this work was used for the first time for a 3D characterization of the selected tumor samples. It thus represented a first step towards the detection of chronic hypoxic areas at the microscopic level for whole tumors. However, direct identification of hypoxic subvolumes, e.g., with the marker pimonidazole, has so far only been possible in 2D, so that the TOM simulations of the oxygen distributions were used to assess these areas.

The TOM simulations revealed low comparability of histological and simulated 3D oxygen parameters, such as the parameter hypoxic distance, due to inherent methodological differences. The simulated oxygen distributions for simplified 2D synthetic capillary grids fit well with the 3D experimental ones for the AT1 tumors. In contrast, a right-shift of the oxygen histograms for the synthetic capillary grids was observed, which occurred due to differences in the spatial capillary distributions and the vessel radius distributions. In addition, a trend toward higher oxygenation after irradiation, i.e., reoxygenation, was evident in the oxygen histograms. This effect was more pronounced in the simulations with synthetic vascular architectures compared to the simulations with experimental ones.

6. Outlook

The mouse tumor xenografts used in this work were also examined with computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound (US) by J. Müller for later evaluation in a radiomics study. This comprehensive data acquisition combined with the findings of J. Müller [114] regarding the 2D histology and this work focused on the LSM microscopy and oxygen simulations, could help bridge the gap between preclinical research and clinical outcome for patients – for example, by back-translating the clinical measurements to the microscopic measurements.

The overall goal could then be to produce a reliable clinical biomarker with the following properties: (i) The marker should describe hypoxia, such as the commonly used positron emission tomography tracers [^{18}F]fluoromisonidazole (FMISO) and [^{18}F]fluoroazomycin-araboside (FAZA), but should (ii) cover additional information about the tumor microenvironment (perfusion, vasculature, etc.). (iii) Furthermore, the biomarker should be measurable contactless, and free of radiation dose, i.e., with magnetic resonance imaging (MRI) or ultrasound (US).

Appendix

A. Deriving a numerical solution of the diffusion-reaction equation

A.1. Fick's laws of diffusion

In 1855, the physiologist Adolf Fick derived the two seminal laws for diffusion processes named after him, based on experimental studies of transport processes in liquids [174].

Fick's first law connects a diffusion flux J with a spatial concentration gradient $d\varphi/dx$:

$$J = -D \frac{d\varphi}{dx}. \quad (\text{A.1})$$

In Equation (A.1), J describes, which amount of a substance with a concentration φ is transported per unit area and unit time along the concentration gradient towards the lower concentration, D is the diffusion coefficient with the dimension area per unit time. With additional consideration of mass conservation in absence of chemical reactions, Equation (A.1) can be rewritten as

$$\begin{aligned} \frac{\partial \varphi}{\partial t} + \frac{\partial}{\partial x} J &= 0 \\ \Rightarrow \frac{\partial \varphi}{\partial t} + \frac{\partial}{\partial x} \left(-D \frac{\partial \varphi}{\partial x} \right) &= 0. \end{aligned} \quad (\text{A.2})$$

Assuming isotropic diffusion and thus a spatially independent diffusion coefficient D , the second term of Equation (A.2) can be expressed as

$$\frac{\partial}{\partial x} \left(-D \frac{\partial \varphi}{\partial x} \right) = -D \frac{\partial}{\partial x} \left(\frac{\partial \varphi}{\partial x} \right) = -D \frac{\partial^2 \varphi}{\partial x^2}. \quad (\text{A.3})$$

Substituting Equation (A.3) into Equation (A.2) yields the formulation of *Fick's second law* as

$$\frac{\partial \varphi}{\partial t} = D \frac{\partial^2 \varphi}{\partial x^2}. \quad (\text{A.4})$$

Equation (A.4) describes the spatiotemporal change in concentration caused by a diffusion process. Assuming an ideal gas, the general gas equation applies and thus the concentration can be replaced by the (oxygen) partial pressure p .

A.2. Approximation of the diffusion-reaction equation using the Krogh-Erlang model

The following two sections closely follow the published description in [84], in which Grimes et al. derived an analytical solution of the DRE from Fick's second law. The derivation in [84] begins with the *diffusion-reaction equation* for a space- and time-dependent oxygen tension $p = p(x, t)$,

$$\frac{\partial p}{\partial t} = D \frac{\partial^2 p}{\partial x^2} - S(p), \quad (\text{A.5})$$

which is obtained by extending Equation (A.4) by a source term $S(p(x, t))$ to account for the oxygen supply through the capillaries.

The Krogh-Erlang model, proposed by August Krogh in 1919 [161], was then used to obtain a fairly simple analytical description of the problem [175]. The model was the first theoretical attempt to understand the oxygen transport into tissue and is based on the following assumptions [176]: The capillaries are homogeneously distributed in the tissue, which is true, for example, for skeletal muscles. Additionally, oxygen diffuses isotropically and is consumed at a constant rate [176]. Axial oxygen transport within the capillary is neglected (and thus that blood is needed as an oxygen carrier) and diffusion occurs only in the direction perpendicular to the capillary [175].

A.3. Radial-symmetric diffusion equation

With the assumptions of the Krogh-Erlang model, the DRE for the radial-symmetric *diffusion profile* can be written for a steady-state problem ($\partial p / \partial t = 0$) as

$$\frac{D}{r} \frac{d}{dr} \left(r \frac{dp}{dr} \right) - a(p)\Omega = 0. \quad (\text{A.6})$$

The parameter r in Equation (A.6) represents the spatial distance to the capillary center, as illustrated in Figure 3.3 b). Equation (A.6) consists of a diffusion and a consumption term $a(p)$ with a constant Ω . The latter may be approximated as in [84] by

$$a(p) = \frac{a_0 p(r)}{p(r) + K_m} \simeq \frac{a_0 (r_c^* - r)^2}{(r_c^* - r)^2 + l_r}. \quad (\text{A.7})$$

In Equation (A.7), the distance measure l_r , defined as

$$l_r = (r_c^* - r_0)^2 \frac{K_m}{p_0}, \quad (\text{A.8})$$

marks the position where the consumption drops to 50 % of the maximum consumption rate a_0 . The parameter K_m indicates the amount of p_{O_2} at the position l_r . The critical diffusion radius r_c^* denotes the distance to the capillary, where the p_{O_2} drops to zero. The approximation in Equation (A.7) makes the consumption independent of the oxygen tension p , and links it instead to the radial distance r , which allows for an

analytical solution. Grimes et al. then used the boundary conditions $p(r_c^*) = p'(r_c^*) = 0$ and $p(r_0) = p_0$ to compute the first derivative of Equation (A.6) with *Mathematica Version 12.0* [162]:

$$p'(r) = \frac{a_o \Omega}{2D} \left(r + \frac{1}{r} \left(2r_c^* \sqrt{l_r} \arctan\left(\frac{(r_c^* - r)}{\sqrt{l_r}}\right) - l_r \ln\left(\frac{(r_c^* - r)^2}{\sqrt{l_r}}\right) - r_c^{*2} \right) \right). \quad (\text{A.9})$$

Finally, integrating Equation (A.9) using $p(r_c^*) = 0$ and $p(r_0) = p_0$ yields the analytical solution of the DRE depicted in Figure 3.3 b).

B. Clearing protocol for LSFM samples

The tumor and brain samples were prepared for scanning with the LSFM using the *iDISCO+ protocol version from December 2016* [123]. The following description closely follows the parts of the original protocol for *whole mouse brains* [123], except for two changes in the preparation of the tissue samples and the clearing highlighted in italics.

B.1. Composition of the buffer solutions

To prevent microbial growth, 0.02 % NaN_3 was added to all stock solutions.

PTx.2 (1 l)

- 100 ml Phosphate-buffered saline (PBS) 10X
- 2 ml TritonX-100

PTwH (1 l)

- 100 ml PBS 10X
- 2mL Tween-20
- 1mL of 10mg/mL Heparin stock solution

Permeabilization Solution (500 ml)

- 400 ml PTx.2
- 11.5g of Glycine
- 100 ml of Dimethyl sulfoxide (DMSO)

Blocking Solution (50 ml)

- 42 ml PTx.2
- 3 ml of Donkey Serum
- 5 ml of DMSO

B.2. Preparation of the tissue samples

1. *Anesthesia of the animals and perfusion were not performed for animal welfare reasons. Instead: Sacrificing the animals by cervical dislocation. [114, p. 70]*
2. Dissection of whole tissue samples from brain or tumor.
3. Additional step: Halving of the tissue samples and either snap-freezing in liquid nitrogen ($-80\text{ }^{\circ}\text{C}$) for 2D histological analysis or fixation in PFA for the subsequent steps. [114, p. 70]
4. Fixation in 1x PBS/4 % PFA at $4\text{ }^{\circ}\text{C}$, overnight with shaking, then *room temperature* (RT) 1 h.
5. Washing in PBS with shaking: RT 30min x 3 times.

The samples were then stored in PBS with 0.02 % NaN_3 at $4\text{ }^{\circ}\text{C}$.

B.3. Methanol pretreatment of the samples

1. Dehydration with methanol/ H_2O series: 20 %, 40 %, 60 %, 80 %, 100 %; 1 h each.
2. Further washing with 100 % methanol for 1 h and subsequent cooling of the sample at $4\text{ }^{\circ}\text{C}$.
3. Overnight incubation, with shaking, in 66 % DCM / 33 % Methanol at RT.
4. Washing twice in 100 % Methanol at RT, and then chilling the sample at $4\text{ }^{\circ}\text{C}$.
5. Bleaching in chilled fresh 5 % H_2O_2 in methanol (1 volume 30 % H_2O_2 to 5 volumes MeOH), overnight at $4\text{ }^{\circ}\text{C}$.
6. Rehydration with methanol/ H_2O series: 80 %, 60 %, 40 %, 20 %, PBS; 1 h each at RT.
7. Washing in PTx.2 RT 1 h, x2 at RT.

B.3. Immunolabeling

All steps were performed in closed, completely filled tubes to prevent oxidation.

1. Incubation of samples in Permeabilization Solution, at $37\text{ }^{\circ}\text{C}$ for 2 days.
2. Blocking in Blocking Solution at $37\text{ }^{\circ}\text{C}$ for 2 days.
3. Incubation with the primary antibody in PTwH/5%DMSO/3% Donkey Serum, at $37\text{ }^{\circ}\text{C}$ for 7days.
4. Washing in PTwH for 4 – 5 times until the next day.
5. Incubation with secondary antibody in PTwH/3% Donkey Serum, 37° , 7 days.
6. Washing in PTwH for 4 – 5 times until the next day.

B.4. Clearing

1. Dehydration in methanol/H₂O series: 20 %, 40 %, 60 %, 80 %, 100 %, 100 %; 1 h each at RT. Can be left optionally overnight at RT at this point.
2. 3 h incubation, with shaking, in 66 % DCM/33 % Methanol at RT
3. Incubation in 100 % DCM (Sigma 270997-12X100ML) 15 minutes twice (with shaking) to wash the MeOH.
4. *Incubation with Ethyl cinnamate instead of DiBenzyl Ether (DBE), no shaking.*

The tube was filled almost completely with the solution to prevent air from oxidizing the sample.

C. Additional histology data

Table C.1. Published data for the two mouse xenografts based on IHC stained histological sections from [114, p. 89]. The VF of different studies diverged but the product of VF and FPV, i.e., the area of the perfused vessels to total viable tumor area was comparable for both. In addition, HF and NF were 50 % higher in [22], which [114] attributed to methodological differences in the classification of the different tumor areas [114, pp. 88–89].

Tumor model	VF (%)	FPV (%)	HF (%)	NF (%)	Source
SAS	3.7 ± 0.6	76.9 ± 10.7	20.5 ± 6.7	46.8 ± 19.2	[22]
	5.3 ± 2.3	41.0 ± 25.1	13.2 ± 4.0	39.0 ± 10.8	[114]
UT-SCC-14	2.2 ± 0.3	83.5 ± 7.6	18.5 ± 6.0	36.3 ± 17.7	[22]
	4.9 ± 2.6	41.7 ± 20.1	12.0 ± 4.9	37.8 ± 18.3	[114]

Abbreviations: VF = vascular fraction, FPV = fraction of perfused vessels (Hoechst33342-positive) to the total amount of vessels (CD31-positive), HF = hypoxic fraction, NF = necrotic fraction.

Table C.2. Additional parameters determined in 2D for histological sections by J. Müller [114] and summarized as part of this work.

Parameter	Unit	SAS			UT-SCC-14		
		Control	Convent.	Hypofract.	Control	Convent.	Hypofract.
N	/	16	19	18	21	17	15
$\bar{d}_{\text{hyp}}$	μm	40 ± 11	47 ± 17	44 ± 13	51 ± 16	42 ± 13	37 ± 10
V_{init}	mm^3	225 ± 71	239 ± 67	225 ± 49	196 ± 51	194 ± 60	179 ± 53
V_{ex}	mm^3	225 ± 71	302 ± 89	279 ± 73	196 ± 51	213 ± 60	211 ± 47
ΔV	mm^3	0	63 ± 40	53 ± 60	0	20 ± 73	32 ± 36
VDT	days	8.9 ± 1.5	8.8 ± 1.4	8.6 ± 1.3	9.7 ± 3.2	11.6 ± 2.8	11.7 ± 2.8
V_{hyp}	mm^3	31.3 ± 20.1	37.8 ± 20.0	33.2 ± 16.2	19.7 ± 12.8	31.8 ± 18.7	24.5 ± 8.5
HF	%	13.0 ± 5.1	12.5 ± 6.6	11.8 ± 4.7	9.9 ± 5.4	14.9 ± 7.0	11.9 ± 4.3
NF	%	52.6 ± 9.8	61.8 ± 9.2	69.7 ± 11.7	56.0 ± 17.1	70.4 ± 7.2	67.9 ± 12.5
VF	%	3.7 ± 1.2	3.0 ± 1.0	3.1 ± 1.3	3.5 ± 1.1	4.3 ± 1.1	4.1 ± 1.4
FPV	%	50 ± 25	57 ± 21	58 ± 19	54 ± 18	53 ± 19	49 ± 15

Abbreviations: N = number of animals, d_{hyp} = distance of a pimonidazole-hypoxic pixel to the next viable vessel (Hoechst33342- and CD31-positive), V_{init} = tumor volume at begin of irradiations, V_{ex} = tumor volume at excision time point, ΔV = volume change $V_{\text{ex}} - V_{\text{init}}$, VDT = volume doubling time, V_{hyp} = pimonidazole-hypoxic volume, HF = hypoxic fraction, NF = necrotic fraction, VF = vascular fraction, FPV = fraction of perfused vessels (Hoechst33342-positive) to the total amount of vessels (CD31-positive).

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Publications

Conference talks

A. Neuholz and J. Müller, "Characterization of vascular damage and hypoxia in experimental HNSCC tumors after photon, proton and carbon ion irradiation to validate bio-mathematical models," *presented at the NCRO Retreat*, Dresden, Germany, 2018.

A. Neuholz, "Modellierung der Gefäßarchitektur und Sauerstoffversorgung in Tumoren sowie Vergleich mit experimentellen Befunden," *presented at the 49. Jahrestagung der Deutschen Gesellschaft für Medizinische Physik & 21. Jahrestagung der Deutschen Sektion der ISMRM*, Nuremberg, Germany, 2018.

A. Neuholz, "Towards experimental validation of a mathematical tumor oxygenation model with an ultra-microscope," *presented at the Workshop Experimental Radiobiology: Physics meets Biology and Medicine*, Santiago, Chile, 2020.

A. Neuholz and J. Müller, "Characterization of vascular damage and hypoxia in experimental HNSCC tumors after photon and proton irradiation to validate bio-mathematical models," *presented at the National Center for Radiation Research in Oncology (NCRO Retreat)*, Heidelberg (Online), 2021.

Poster presentations

A. Neuholz, "Modeling of vascular architecture & oxygen distribution in tumors & comparison with experimental findings," *presented at the DeGBS Annual Conference 2019 der Deutschen Gesellschaft für Biologische Strahlenforschung e.V.*, Mannheim, Germany, 2019.

A. Neuholz, "Simulation der 3D-Gefäßarchitektur und Sauerstoffverteilung von Plattenepithelkarzinomen des Kopfes und Halses (HNSCC) und Vergleich mit experimentellen Befunden," *presented at the 51. Jahrestagung e.DGMP der Deutschen Gesellschaft für Medizinische Physik*, Leipzig (Online), Germany, 2020.

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