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Julian Dominik Rickert

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3D-4Pi MINFLUX nanoscopy

Referees: Prof. Dr. Dr. h. c. mult. Stefan W. Hell
Prof. Dr. Joachim Spatz

Zusammenfassung

Die Bestimmung der Position von fluoreszenten Emittern in allen drei räumlichen Dimensionen und der zeitlichen Dimension ist ein wichtiges Werkzeug in den Biowissenschaften, weil dadurch dynamische Prozesse in biologischen Strukturen höchst präzise und minimal invasiv untersucht werden können. Die zu erreichende räumlich-zeitliche Auflösung solcher Untersuchungen ist üblicherweise limitiert durch die Rate an emittierten Photonen. In dieser Arbeit wird sowohl theoretisch als auch im Experiment gezeigt, dass in einem MINFLUX-Setup mit einem 4Pi-Interferometer diese Ressource mittels kohärent interferierender gegenläufiger Strahlen bestmöglich genutzt werden kann. Isotrope 3D Präzision wird erreicht, indem die gegenläufigen Strahlen im Fokus verkippt werden. Um 3D MINFLUX Messungen zu ermöglichen, wurden ein Phasenscanner und ein Strahlscanner entwickelt und in einer 4Pi-Optik implementiert. Ersterer ermöglicht es ein interferometrisch erzeugtes lokales Minimum des Anregungslasers entlang der Propagationsachse der Laserstrahlen zu verschieben. Letzterer erlaubt die Positionierung des Laserstrahls in der Pupille der Mikroskopobjektive, wodurch die Messachse verkippt wird. Beide Scanner beruhen dabei auf Kristalloptik, so dass sie mit höchster räumlicher und zeitlicher Präzision elektrooptisch angesteuert werden können. Mit dem hier verwendeten MINFLUX-Mikroskop werden Messungen an Einzelmolekülen mit einer um 40 % höheren Photoneneffizienz als bei bisherigen 3D-MINFLUX Messungen gezeigt. Zudem wird an hellen, photostabilen Emittern sub-nm Präzision auf kurzen Zeitskalen bei einer Messrate von ca. 9 kHz pro Achse demonstriert. Die hohe räumlich-zeitliche Auflösung des Systems wird dazu verwendet, um neue Einsichten in das Schrittverhalten von DNA-Nanorobotern und die 3D Diffusion von Einzelmolekülen und fluoreszierenden Kügelchen zu erlangen. Die erzielten Ergebnisse zeigen die Überlegenheit eines 3D-4Pi-MINFLUX Mikroskops bei der Verfolgung von einzelnen fluoreszierenden Emittern in drei Dimensionen.

Abstract

Determining the position of fluorescent emitters in all three spatial dimensions and the temporal dimension is an essential tool in the life sciences because it allows dynamic processes in biological structures to be studied with high precision and minimal invasiveness. The rate of emitted photons usually limits the achievable spatio-temporal precision of such investigations. In this work, it is shown both theoretically and experimentally that in a MINFLUX setup with a 4Pi interferometer, this resource can be optimally leveraged by using coherently interfering counterpropagating beams. 3D isotropic precision is achieved by introducing tilts to these beams in the focal region. To enable 3D MINFLUX measurements, a phase scanner and a beam scanner have been developed and implemented in a 4Pi optics. The former allows for shifting an interferometrically generated local minimum of the excitation laser along the propagation axis of the laser beams. The latter allows positioning the laser beam in the pupil of the microscope objectives, tilting the measurement axis. Both scanners are based on crystal optics, so they can be controlled electro-optically with the highest spatial and temporal precision. With the MINFLUX microscope used here, measurements on single molecules are shown to have a 40 % higher photon efficiency than previous 3D MINFLUX measurements. In addition, sub-nm precision at short time scales on bright, photostable emitters at a measurement rate of approximately 9 kHz per axis is demonstrated. The high spatio-temporal precision of the system is used to gain new insights into the stepping behavior of DNA nanorobots and the 3D diffusion of single molecules and fluorescent beads. The results obtained demonstrate the superiority of a 3D-4Pi MINFLUX microscope in tracking single fluorescent emitters in three dimensions.

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List of Abbreviations

1D	one dimension
2D	two dimensions
3D	three dimensions
4Pi-SMS	4Pi single molecule switching
AOD	acousto-optical deflector
AOTF	acousto-optic tunable filter
APD	avalanche photodiode
BFP	back focal plane
BIC	Bayesian Information Criterion
BSC	beam splitter cube
COT	Cyclooctatetraene
dc	direct current
DDS	direct digital synthesizer
DM	dichroic beam splitter
DNA	deoxyribonucleic acid
DNA PAINT	deoxyribonucleic acid points accumulation for imaging in nanoscale topography
EOM	electro-optical deflector
FCE	Fixed Curvature Estimator
FCS	fluorescence correlation spectroscopy
FPGA	field-programmable gate array
FRAP	fluorescence recovery after photobleaching
FWHM	full width at half maximum
GL	Glen Laser polarizer
GNP	gold nanoparticle
GNP-bt	biotin gold nanoparticle
GSD	ground state depletion
iSCAT	interferometric scattering microscopy
$\lambda/2$	half-wave plate
LP	long pass filter
LSM	light sheet microscopy
mechBB	mechanical beam block
mot$\lambda/2$	motorized half-wave plate
mot$\lambda/4$	motorized quarter-wave plate
motPH	motorized pinhole
MSD	mean squared displacement
NA	numerical aperture
NVD	Neutravidin
OT	orbital tracking

PAINT	points accumulation for imaging in nanoscale topography
PALM	photoactivated localization microscopy
PBS	phosphate buffered saline
PBSC	polarizing beam splitter cube
PEG	Polyethylene Glycol
PLL	Poly-L-Lysin
PMT	photomultiplier tube
PSF	point spread function
RESOLFT	reversible saturable optical fluorescence transitions
RF	radio frequency
RTP	rubidium titanyl phosphate
SBR	signal-to-background ratio
SMF	single-mode fiber
SPT	single particle tracking
ssDNA	single-stranded deoxyribonucleic acid
SSR	sum of squared residuals
STED	stimulated emission depletion
STORM	stochastic optical reconstruction microscopy
STP	spatio-temporal precision
TAE	Tris-acetate-EDTA
TDE	2,2'-thiodiethanol
TIRF	total internal reflection fluorescence
VHDL	Very High Speed Integrated Circuit Hardware Description Language
VFL	varifocal lens

Chapter 1

Introduction

Light microscopy in life sciences

In its pursuit to understand evermore about the natural world, humankind has developed numerous sophisticated measuring instruments. The far-field optical microscope aims to resolve and understand the smallest structures and processes in biological and living matter. Paired with the fluorescence of single fluorescent molecules, the far-field microscope offers a non-invasive, highly specific, and high contrast detection method [1]. Additionally, the photophysical properties of different fluorescent molecules can be used to image several structures simultaneously [2]. Finally, the spatial and temporal localization precision of fluorescent microscopes has constantly been improving over the last century [3].

Fluorescence microscopy is used for spatially resolving biological structures like cell surfaces, vesicles, nuclear pores, and the neuronal cytoskeleton, among other things [4]. Furthermore, the technique is applied to obtain insights into various dynamical processes, including but not limited to movements driven by molecular motors, stochastic processes, and movement in confined spaces [5–8]. Due to fluorescent microscopes' spatial and temporal localization precision constantly improving, more and more of the structures and dynamics governing our life could be revealed [3].

The diffraction limit

Until the end of the 20th century, the diffraction limit was thought to be the limiting factor for localization precision in fluorescence microscopy. It was formulated in 1873 by the physicist Ernst Abbe [9] and is based on the assumption that the diffraction of electromagnetic waves is the limiting factor in spatial precision. Diffraction leads to an

Airy pattern [10] in a focused light beam, with the first dark ring appearing at

$$r_{lateral} = \frac{0.61\lambda}{NA} \quad (1.1)$$

$$r_{axial} = \frac{2n\lambda}{NA^2} \quad (1.2)$$

Here, λ is the wavelength of the light in vacuum, n the immersion medium's refractive index, and $NA = n \sin(\delta)$ the numerical aperture of the optical system with δ being the half angular aperture of the optical system. Therefore, by maximizing the NA, the diffraction limit could be lowered, but the problem of inferior axial precision, which is about a factor 3-4 worse than lateral precision, remained, leaving a lateral precision of ~ 200 nm and an axial precision of ~ 700 nm for $\lambda = 500$ nm.

In the realm of diffraction-limited microscopy, several techniques have been invented to increase the axial precision; these include techniques such as confocal microscopy, light sheet microscopy (LSM) [11], multiphoton excitation [12] or total internal reflection fluorescence (TIRF) [13]. These techniques make use of different concepts, such as filtering with a pinhole, illuminating only a single plane, optical non-linearities, and evanescent waves. None of these techniques achieves an axial precision below 100 nm [14], except for TIRF, which can reach up to 20 nm axial precision [15] but is limited to an imaging depth of only 100-200 nm.

Dual-objective systems with coherent illumination (4Pi) [16] marked an improvement towards isotropic 3D precision, as they demonstrate a sevenfold improvement in axial localization precision [17], without sacrificing lateral precision. These systems use a second objective opposing the first objective to illuminate the sample from both sides and effectively increase the angular aperture of the optical system by a factor of two. While providing 3D isotropic measurements, these systems have the caveat that a high effort needs to be put into the calibration to ensure diffraction-limited operation.

Conventional Nanoscopy

Diffraction-unlimited microscopy emerged in the 1990s from the idea to utilize the on-off switching of fluorophores between a fluorescent (on) and a non-fluorescent (off) state combined with an optical setup that ensures that only one fluorophore is in the on-state inside of a diffraction-limited region at the time of observation.

In 1994, Prof. Stefan Hell was the first scientist to postulate that diffraction-unlimited fluorescence microscopy is possible by targeted deactivation of fluorophores via stimulated emission [18]; he later moved on to prove his assumptions by demonstrating the first superresolution microscope [19], called stimulated emission depletion (STED). Other

superresolution techniques such as ground state depletion (GSD) [20] and reversible saturable optical fluorescence transitions (RESOLFT) [21] work with the same principle. As the idea is to de-excite fluorophores via stimulated emission everywhere but at a confined intensity minimum of the de-excitation laser, these methods are called coordinate-targeted superresolution. The de-excitation effectively narrows the area of still-excited fluorophores down to [22]

$$\sigma_{STED} \propto \frac{\sigma_{PSF}}{\sqrt{1 + I/I_S}}. \quad (1.3)$$

Here, I is the intensity of the de-excitation beam, I_S is the saturation intensity of the fluorophores, i.e., the intensity at which a fluorophore goes back to the de-excited state with a probability of 50%, and σ_{PSF} is the $1/\sqrt{e}$ -radius of the excitation beam.

In 2006, scientists from the United States devised another superresolution scheme based on the subsequent localization of stochastically switched molecules, called stochastic superresolution techniques. The most prominent of these methods are known as photo-activated localization microscopy (PALM) [23], stochastic optical reconstruction microscopy (STORM) [24], and points accumulation for imaging in nanoscale topography (PAINT) [25]. These techniques typically utilize a camera detector, on which each fluorophore imprints a diffraction-limited signal. By collecting N fluorescence photons from a single fluorophore and again ensuring that only a single fluorophore is switched on in a diffraction-limited region during the time of a single measurement, the position of the fluorophore can be estimated with sub-diffraction precision to be

$$\sigma = \frac{\sigma_{PSF}}{\sqrt{N}}. \quad (1.4)$$

The coordinate-targeted, as well as the stochastic methods, could push the lateral localization precision well below the diffraction limit. However, pushing the axial limit needed additional effort. In stochastic methods, the illumination function has been manipulated to imprint axial information in the shape of the fluorophore's diffraction spot on the camera detector. These methods include astigmatic point spread functions (PSF) [26] and double helix PSF [27]. In coordinate-targeted approaches, axial superresolution could be achieved by generating a minimum of the de-excitation laser along the optical axis [28]. These advances also pushed the axial precision below the diffraction limit, but it was still worse than the lateral precision.

Isotropic precision could be achieved using a 4Pi configuration, which has been demonstrated in various superresolution modalities. isoSTED [29] is the combination of STED with a 4Pi optics; other examples are 4Pi STORM [30], iPALM [31], and 4Pi single molecule switching (4Pi-SMS) [32].

Measuring dynamics in light microscopy

In addition to providing images of biological structures, fluorescence microscopy is also used to reveal information about the dynamics of labeled objects. Information can be obtained from ensembles, as is done in fluorescence correlation spectroscopy (FCS) [33] and fluorescence recovery after photobleaching (FRAP) [34] or from single particle tracking (SPT) [5, 35], which provides information on the single particle level. SPT techniques can be divided into widefield illumination methods such as sptPALM [36], continuous point illumination methods such as orbital tracking (OT) [37], and discrete point illumination [38, 39]. These techniques achieve a lateral and axial precision of below 10 nm and below 50 nm respectively at a temporal precision of about 30 ms [37].

For investigating dynamics, temporal precision is as important as spatial precision. Therefore, scattering emitters such as gold nanoparticles (GNP) with sizes of 20 nm up to several μm are preferred because the rate of scattered photons can be much higher than the rate of fluorescence from single fluorescent molecules or fluorescent beads, as it only depends on the intensity of the excitation laser. These emitters have been used in widefield setups such as interferometric scattering microscopy (iSCAT) [40] and in confocal setups [41], where 3D single-digit nanometer precision was obtained in sub-100 μs .

In general, scatterers provide a higher spatio-temporal precision (STP), but they are larger than single fluorescent molecules and prone to induce phototoxicity due to heating. Therefore, methods employing large scatterers are unsuited if a high labeling accuracy is required or if the size of the particle influences the observed properties.

The practical limits of conventional nanoscopy

Spatial and temporal precision is enhanced by collecting information from the sample. In fluorescence microscopy, this information is obtained from the fluorophores undergoing fluorescence cycles and emitting photons toward a detector. Despite efforts to increase the photon budget from a single fluorophore, photobleaching limits the practically achievable localization precision of coordinate-targeted and stochastic superresolution methods to $\sim 20\text{-}30\text{ nm}$ [42]. Although theoretically possible, single-digit precision is rarely achieved, an example being deoxyribonucleic acid PAINT (DNA PAINT) [43], where the information for a single localization was gathered from more than one fluorophore. Nevertheless, increasing the number of available fluorescence photons using more stable fluorophores or using multiple fluorophores has not led to molecular precision

in conventional nanoscopy methods. In addition, using many fluorescence photons for gaining accurate spatial precision is at the expense of temporal precision. This problem originates from the inefficient use of fluorescence photons; Equations 1.3 and 1.4 show that the information gained from each additional photon scales inversely with the root of the photons and thus stagnates for large photon numbers. For a more efficient use of the limited photon budget, a more flexible method is needed, where the information of the first measured photons is used to exploit the information content of subsequent photons.

MINFLUX - Breaking the practical limits of conventional nanoscopy

Recently, a new superresolution technique using the limited fluorescence photon budget of single fluorophores more efficiently than any previous method was introduced under the name of MINFLUX [44]. MINFLUX nanoscopy combines the properties of stochastic and coordinate-targeted superresolution to resolve fluorophores with nanometer precision using just a few fluorescence photons. For this purpose, as in stochastic nanoscopy, it is first ensured that only one fluorophore in a diffraction-limited region is in the on-state. Then, as in coordinate targeted superresolution, the fluorophore is excited with a focused illumination beam whose position is precisely known. In addition, the illumination beam has a local intensity minimum, where the goal is to place this minimum as close as possible to the position of the fluorophore. In theory, the position of the fluorophore is exactly known if it coincides with the position of the minimum, and thus no photon is emitted. In practice, the position of the fluorophore must first be narrowed down so that the minimum of the illumination can approach its position more and more. For this, the minimum of the illumination is driven in a pattern around the fluorophore. Then the minimum is moved closer and closer to the position of the fluorophore in an iterative process based on the collected information. Using MINFLUX, the $\sigma \propto 1/\sqrt{n}$ law of conventional nanoscopy is replaced by an exponential information gain $\sigma \propto e^{-N}$ [45]. This makes each photon equally informative and maximizes the information gain per photon, referred to as photon efficiency in this work.

Since the introduction of MINFLUX, other techniques promising similar results have been developed. However, most of the methods claiming to be similar to MINFLUX [46–51] do not overcome the square-root law of conventional nanoscopy, as they do not provide the flexible illumination scheme which is needed for MINFLUX. MINSTED is the only other recently developed technique that accomplishes the same feat of breaking the square-root law. It has been demonstrated to achieve sub-nanometer precision [52].

MINFLUX can achieve molecular precision using less than 1000 photons and has demonstrated an exceptional STP in tracking applications [53]. Furthermore, the method has already been extended to live-cell, multicolor, 3D measurements [54–56]. Also, it was used

for imaging encapsulins [57], resolving the photoreceptor active zone [58], imaging bacterial molecular machines [59], and investigating the walking mechanism of kinesin [45, 60]. Recently, it has been combined with PAINT to three-color images of mitochondria [61]. Until now, 3D MINFLUX measurements have been performed in single-objective setups only. Therefore, isotropic 3D precision could only be achieved with a significantly higher photon cost for localization along the optical axis than in the lateral plane. The best 3D MINFLUX measurements to date demonstrate a localization precision of 2.0 nm with 400 photons per dimension [56] in imaging measurements and an STP of 9.5 nm at a detection rate of 2.1kHz for tracking measurements [62].

None of the setups has used 4Pi optics, which generates the best axial illumination function. Additionally, the movement of the intensity minimum along the axial direction was induced by an electro-optically actuated varifocal lens (VFL) [54] or a deformable mirror [56], which are an order of magnitude slower compared to the electro-optics, which is used to position the intensity minimum along the lateral plane.

Motivation and structure of this work

This work aimed to improve the STP and photon efficiency of 3D MINFLUX tracking measurements by using a 4Pi setup combined with a fast beam scanning approach. To this end, an existing 4Pi setup was modified to use a phase scanner and a beam scanner to modulate the position of the intensity minimum at the focus of the two objectives within a few μ s.

The beam and phase scanner were developed and implemented as part of this thesis, with particular attention being paid to meeting the challenge of producing a good intensity minimum. It should be noted that this is a major challenge in a 4Pi setup due to residual optical aberrations and is of great importance for the MINFLUX approach, as the maximum achievable localization precision scales with the residual intensity at the minimum. A sophisticated alignment procedure combined with the flexible beam scanning enabled by the acousto-optical components implemented for the beam scanner could alleviate this problem. As a result, the theoretical superiority of a 4Pi configuration translates into experimental performance by showing the highest photon efficiency ever measured for single fluorescent molecules despite the difficulties imposed by a 4Pi setup.

In Chapter 2 of this thesis, the theory of MINFLUX nanoscopy is presented, the transformation from the 4Pi coordinate system to the laboratory coordinate system and its influence on the localization precision is discussed, and it is shown that a 4Pi configuration theoretically achieves the best 3D localization precision of any illumination configuration.

In Chapter 3, the 3D-4Pi MINFLUX setup is explained in detail, with special emphasis on the phase scanner and beam scanner and how they contribute to a MINFLUX measurement. This chapter explains how the setup is adjusted and how a position in the laboratory system is retrieved from a 3D-4Pi MINFLUX measurement.

Chapter 4 summarizes the tracking measurements. 3D-4Pi MINFLUX shows about 40% better photon efficiency as the best 3D MINFLUX measurement demonstrated before. In addition, it shows the highest STP at bright, stable emitters by locating them at a rate of 2.9 kHz per 3D localization with sub-nm precision at short timescales. This corresponds to a precision of <1 nm in less than $100 \mu\text{s}$ per dimension.

In addition, the 3D tracking accuracy of the assembly was tested on DNA nanorobots, reproducing the expected behavior in the lateral direction and showing that no significant motion occurs in the axial direction. Finally, a study of freely diffusing single fluorescent molecules and fluorescent beads in highly viscous environments was performed. This study shows that diffusion at the nanoscale is orders of magnitude faster than diffusion on scales > 50 nm.

Chapter 2

Theoretical Framework

In this chapter, the theory behind MINFLUX nanoscopy, in general, and 3D-4Pi MINFLUX, in particular, is described in detail. Section 2.1 elaborates on the general idea behind MINFLUX and demonstrates how it overcomes the square-root law of previous superresolution techniques. Also, the performance metrics of photon efficiency and spatio-temporal resolution (STP) are quantified. Section 2.2 shows how the choice of illumination pattern affects the localization precision of a 3D measurement in a 4Pi-MINFLUX setup. Section 2.3 concludes the chapter with a comparison of the achievable localization precision in single-objective MINFLUX and 4Pi-MINFLUX, showing that 4Pi outperforms single-objective by a factor of 1.5.

2.1 MINFLUX - A new Paradigm in Superresolution Microscopy

MINFLUX combines a coordinate-targeted localization scheme with the on-off switching mechanism of certain fluorophores, enabling a photon efficiency beyond the inverse square-root law achieved in conventional superresolution techniques. This is accomplished by addressing each fluorophore with a sequence of individual exposures exhibiting a local intensity minimum. Here, the intensity minimum is shifted within an area of size L around the fluorophore. Then, the position of the fluorophore is estimated from the individual photon counts and the positions of the local intensity minimum. The unparalleled photon efficiency achieved in MINFLUX nanoscopy stems from an iterative application of the localization sequence mentioned above and the optimal contrast in detected photons when the excitation pattern is centered around the fluorophore position. The iterative localization approach allows for an adaption of the localization sequence given the information already obtained in the previous iteration. Below, the derivation for the maximally achievable

localization precision for a MINFLUX measurement with three equidistant exposures along one dimension will be outlined, as this localization scheme is applied throughout this thesis.

A single MINFLUX cycle

Most illumination intensities exhibiting a local minimum can be approximated by a parabola around their minimum

$$I(x) = a + b(x - x_e)^2 \quad (2.1)$$

with a being an offset, b the curvature of the parabola, and x_e the position of an emitter relative to the intensity minimum. Assuming that the intensity is below the saturation intensity of the illuminated emitter, it will emit a number of fluorescence photons proportional to the illumination intensity

$$N \propto I(x_e). \quad (2.2)$$

Therefore, information about the position of the emitter x_e can be extracted from the photon numbers collected during the measurement.

A measurement of the emitted photons for a single exposure is not enough to determine the position of the emitter as the parameters a and b are unknown because they depend on a multitude of parameters, such as the laser intensity, the background in the sample and the quantum yield of the fluorophore, to only name a few. In order to extract all three parameters, each MINFLUX measurement consists of a series of three exposures at positions $x = (-L/2, 0, L/2)$ yielding the photon counts

$$\begin{aligned} n_- &= b(x_e - L/2)^2 + a \\ n_0 &= bx_e^2 + a \\ n_+ &= b(x_e + L/2)^2 + a \end{aligned} \quad (2.3)$$

This system of equations can be solved for the emitter position and gives

$$x_e = \frac{L}{4} \frac{n_+ - n_-}{n_+ + n_- - 2n_0} \quad (2.4)$$

The error on this estimate can be derived via gaussian error propagation and assuming poissonian photon noise it is given by

$$\sigma = \frac{L}{2} \frac{\sqrt{(n_+ - n_-)^2 n_0 + (n_+ - n_0)^2 n_- + (n_0 - n_-)^2 n_+}}{(n_+ + n_- - 2n_0)^2}. \quad (2.5)$$

If the illumination pattern is centered around the emitter position and the background is zero, i.e. $x_e = a = 0$, the error simplifies to

$$\sigma = \frac{L}{4\sqrt{N}} \quad (2.6)$$

where $N = n_- + n_0 + n_+$ is the total number of detected photons. Experimentally more relevant is the case for non-zero background, i.e. $a \neq 0$. Here, the signal-to-background ratio (SBR) can be defined as

$$\text{SBR} := \frac{n_+ + n_- - 2n_0}{2n_0} = \frac{bL^2}{4a}. \quad (2.7)$$

By this definition, the photons from the central exposure are the background photons, and the signal photons are the photons collected during the outer exposure minus the background photons.

Inserting Equations 2.3 into Equation 2.5 and substituting N and SBR one gets another expression for the localization precision in the case of non-zero background:

$$\sigma = \frac{L}{4\sqrt{N}} \sqrt{1 + \frac{5}{2\text{SBR}} + \frac{3}{2\text{SBR}^2}} \quad (2.8)$$

Equation 2.8 shows that the localization precision cannot be improved indefinitely by choosing a small L in a measurement with a non-zero background because the SBR decreases quadratically with smaller L . It has been shown [63] that a minimally achievable localization precision of

$$\sigma_{\min} \propto \sqrt{\frac{a}{bN}} \quad (2.9)$$

can be achieved at a minimal L where $\text{SBR} = \sqrt{3/2}$. Therefore, a MINFLUX illumination scheme should be optimized to feature a small intensity minimum a and a high curvature b .

Iterative MINFLUX

Up to this point, only a single MINFLUX measurement cycle has been discussed. From Equations 2.6, 2.8, and 2.9, it becomes evident that this single MINFLUX cycle is still limited by the inverse square root law similar to other superresolution techniques. In contrast, MINFLUX makes more efficient use of the rapid increase in localization precision for small photon numbers by iteratively minimizing L upon the information from the previous measurement cycle.

This iterative procedure is implemented in the measurements presented throughout this thesis as a series of three measurement cycles with a predefined decrease in L . However, a more photon efficient but computationally more demanding procedure exists. The derivation described below again assumes that no background is present. Detecting a single photon from the emitter determines its position within the diffraction limit σ_0 . Then, an iterative MINFLUX measurement can be performed, where

$$L_i = \eta \sigma_{i-1} \quad (2.10)$$

with η being a parameter to balance the decrease in L and the number of emitters staying within the area of the excitation pattern. Performing the measurement n times results in a localization precision

$$\sigma_n = \sigma_0 \left(\frac{\eta}{4} \right)^n \frac{1}{\sqrt{\prod_{i=1}^n N_i}} \quad (2.11)$$

with N_i being the number of photons detected in the i th step. This expression becomes minimal for a given number of detected photons when all N_i are equal and when $\partial \sigma_n / \partial n = 0$, which is fulfilled for $n = 16 \sum_i N_i / (\eta^2 e)$. Then,

$$\sigma = \sigma_0 \left(\frac{\eta}{4} \right)^{16N/(\eta^2 e)} e^{-16N/(\eta^2 e)} \quad (2.12)$$

where $N = \sum_i N_i$ is the number of detected photons throughout the localization process. Therefore, MINFLUX replaces the square-root dependency of conventional superresolution microscopy with an exponential precision increase. As a result, it is the first method to maximize the information content of each individual photon. To put it another way, with MINFLUX, each additional photon results in the same information gain as the first, whereas with previous super-resolution methods, the photons following the first photon contribute less and less to the localization precision.

Fixed Curvature Estimator

The estimator given in Equation 2.4 is well suited for rapid computation of the current emitter position, which is why it is used during a MINFLUX measurement sequence. On the other hand, for a series of M measurements with identical parameters, one can make additional assumptions to obtain a more robust estimator. Assuming a constant illumination and fluorescence response of the emitter, one can calculate the mean curvature

$$\bar{b} = \frac{2}{M} \sum_{i=1}^M \frac{n_{+,i} + n_{-,i} - 2n_{0,i}}{L^2}. \quad (2.13)$$

Inserting the average detected photon numbers to cancel the sum and substituting the expression for the denominator in Equation 2.4 yields the Fixed Curvature Estimator (FCE)

$$x_e = \frac{n_+ - n_-}{2bL}. \quad (2.14)$$

This estimator is handy for tracking measurements at low photon numbers, as the assumptions are well met, and the robustness of the position estimation increases considerably for a few photons, as discussed in [63].

Performance quantification via photon efficiency

The localization precision is often regarded as a performance metric in superresolution microscopy. This notion disregards the influence of emitter properties, such as the total number of emitted photons, which is scarce in fluorescence microscopy. Therefore, the photon efficiency is demonstrated as a different measure for localization performance.

As statistics dictates the uncertainty of the average of a series of N independent measurements to be

$$\sigma = \frac{\Sigma}{\sqrt{N}}, \quad (2.15)$$

with Σ being the uncertainty of a single measurement, it is reasonable to use this formula for determining the performance of a microscopy localization scheme. Given the achieved localization precision σ and the number of photons measured to achieve this localization precision results in an estimate for the photon efficiency Σ .

For PALM/STORM, the optimum achievable photon efficiency is the diffraction limit $\Sigma \equiv \sigma_{\text{PSF}}$, whereas, for a single MINFLUX iteration, it is given as $\Sigma \equiv \sigma_L = L/4$. In later chapters, the photon efficiency calculated from a MINFLUX measurement will be referred to as σ_L .

Spatio-temporal precision

In microscopy, the temporal precision is as vital as the localization precision as it determines the speed at which an image can be acquired and the shortest timescale at which dynamics can be observed in a tracking measurement. The spatio-temporal precision (STP) of a tracking measurement can be defined as

$$\text{STP} = \sigma\sqrt{t} \quad (2.16)$$

with the localization precision σ and the time of a single measurement t . Substituting the emission rate $R = N/t$ and the general expression for localization precision from Equation 2.15 reveals

$$\text{STP} = \Sigma \sqrt{R^{-1}}. \quad (2.17)$$

Therefore, the STP solely depends on the photon efficiency of the measurement and the emitter's fluorescence rate. Hence, given an emitter with a fixed fluorescence rate, MINFLUX can achieve the highest STP as the localization precision scales exponentially with the number of detected photons (s. Equation 2.12). The advantage of MINFLUX is even more pronounced in tracking applications, as the iterative minimization of L only needs to be performed once, and all consecutive measurements can be performed with the smallest L providing a maximal photon efficiency.

2.2 3D-4Pi MINFLUX

A 4Pi MINFLUX system combines the outstanding photon efficiency of the MINFLUX concept and the narrow axial illumination intensity distribution of a 4Pi microscope. Therefore, it can be utilized to perform high-precision 1D measurements along the optical axis, as has been demonstrated in [64]. Here, it will be shown how a particular illumination configuration in a 4Pi setup can be exploited to perform 3D MINFLUX measurements and how this configuration influences the maximum photon efficiency.

The local intensity minimum in 4Pi MINFLUX is generated by the destructive interference of two counterpropagating laser beams entering the sample through two opposing objectives. This is in contrast to the single objective case, where all beams are directed to the sample from a single side. The formation of the focal intensity distribution can be understood as interference of plane waves which results in an intensity distribution

$$I(x) \propto \sin^2 \left(\frac{2\pi n \sin(\phi) x}{\lambda_0} \right) \stackrel{\text{Taylor}}{\approx} \frac{4\pi^2 n^2 \sin^2(\phi)}{\lambda_0^2} x^2 := \frac{\pi^2}{\Lambda^2} x^2 \equiv b x^2. \quad (2.18)$$

Here, ϕ is half the angle between the two interfering waves, and $\lambda = \lambda_0/n$ is the wavelength in the medium. Furthermore,

$$\Lambda := \frac{\lambda_0}{2n \sin \phi} \quad (2.19)$$

is the wavelength of the interferometrically generated illumination profile. The Taylor expansion around the intensity minimum reveals that $b \propto 1/\Lambda^2$. Plugging this expression

into Equation 2.9 shows that

$$\sigma_{min} \propto \Lambda. \quad (2.20)$$

Therefore, the maximally achievable photon efficiency is proportional to the illumination profile's wavelength. Equation 2.18 gives the lateral intensity distribution for a single objective illumination and the axial intensity distribution for a 4Pi illumination with two counterpropagating waves. Due to the counterpropagating waves in 4Pi, $\phi = 90^\circ$, leading to the smallest possible illumination profile wavelength given a fixed excitation wavelength. The lateral illumination profile wavelength in a single objective configuration is always limited by the objective's finite aperture, which prevents a ϕ of 90° .

For performing 3D measurements in a 4Pi configuration, the counterpropagating beams need to be tilted to the optical axis such that measurements can be performed along oblique axes while still utilizing the same optimal curvature as for measurements along the optical axis.

In this implementation, a 3D position is obtained by measuring the position of an emitter consecutively along three oblique axes. This process is demonstrated in Figure 2.1. In principle, the oblique axes can be chosen arbitrarily as long as no two axes are parallel. If the axes are forming a pattern, where they are equidistantly spaced on a circle of radius r around the optical axis, an analytical expression for the coordinate transformation and the resulting σ can be obtained. A detailed derivation is given in Appendix A. Here, only the most important results are presented.

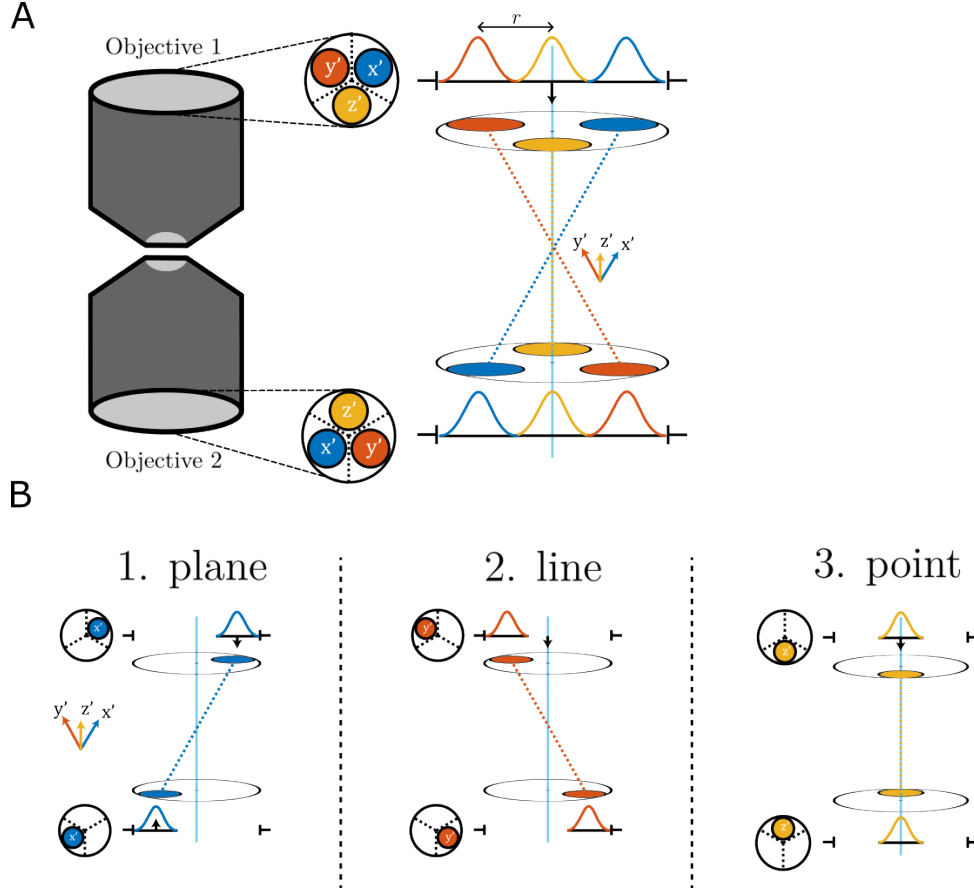


Figure 2.1: **Concept of a 3D measurement in a 4Pi system:** **A.** Axes of propagation (x', y', z') for three pairs of beams, which are displaced from the optical axis at their respective objective's pupil such that each beam pair is non-parallel to the other pairs. Here, the beams are arranged in a pattern where they are equidistantly placed on a circle with radius r around the optical axis. **B.** A 3D measurement begins with a measurement along the x' axis determining the emitter position within a plane perpendicular to this axis. Then, a measurement along the y' axis narrows the position down to a line perpendicular to y' and x' . Finally, a third measurement along the z' axis determines the point at which the emitter sits in the (x', y', z') beam coordinate system. Figure adapted from [64].

An oblique axis can be addressed by displacing the beams from the optical axis such that the shift of the beam through one objective is the point reflection of the shift of the beam through the second objective, i.e., $(\Delta x, \Delta y)$ becomes $(-\Delta x, -\Delta y)$. For a lateral displacement of r from the optical axis in the back focal plane (bfp), the new axis will form an angle of

$$\delta = \arcsin \left(\frac{r}{f \cdot n} \right) \quad (2.21)$$

with the optical axis. Here, f is the focal length of both single objectives, and n is the medium's refractive index. For an angle of $\delta = 54.7^\circ$, the three beams form a cartesian coordinate system, i.e., the angle between each pair of axes is equal to 90° . For such a large angle, the beams need to be placed close to the edge of the objective aperture,

where residual aberrations are more pronounced. Therefore, in practice, the angle between the beam axes is smaller than 90° . The shearing angle β is a measure of how much the coordinate system spanned by the three oblique axes is sheared relative to a cartesian coordinate system.

The relationship between δ and β is:

$$\beta = \arctan \left(\frac{4 - 3 \cdot \sqrt{1 - \cos 4\delta}}{12 \cos(\delta)^2 - 8} \right) \quad (2.22)$$

The angles δ and β are given in Figure 2.2 as a function of the radius of the beam pattern in the bfp. A decrease in the pattern radius leads to an approximately linear decrease in the angle of the individual beams with the optical axis. As most pattern radii lead to non-cartesian coordinate systems, a coordinate transformation into the cartesian laboratory system must be performed to obtain the actual 3D position of a measured object.

According to the transformation given in Appendix A, uncertainties in the beam system propagate to the laboratory system. Under the assumption that the uncertainty along all three measured axes is equal to σ_{beam} , the uncertainty in the laboratory system is given as:

$$\sigma_x = \sigma_y = \sigma_{beam} \cdot \sqrt{\frac{\cos(\beta) \sqrt{1 + 2 \tan(\beta)}}{3 - 6 \cos(\beta) \sin(\beta)}} := \sigma_{beam} T_{lat}(\beta) \quad (2.23)$$

$$\sigma_z = \sigma_{beam} \cdot \frac{\cos(\beta) \sqrt{1 + 2 \tan(\beta)}}{\sqrt{3}(\cos(\beta) + 2 \sin(\beta))} := \sigma_{beam} T_{ax}(\beta) \quad (2.24)$$

Here, T_{lat} and T_{ax} are the factors influencing the localization precision in the laboratory system based on the geometry of the beam system. These relationships are plotted in Figure 2.2. For a shearing angle of $\beta = 0^\circ$, all axes will have the same σ , which is equal to σ_{beam} . The larger β , the more the beam system is sheared towards the optical axis leading to a more accurate σ_z while $\sigma_{x/y}$ are diminished.

The 3D localization precision can be expressed by the detection volume, which is given as

$$\sigma_{geo} = \sqrt[3]{\sigma_x \sigma_y \sigma_z} \quad (2.25)$$

In general, the detection volume becomes optimal when $\beta = 0$.

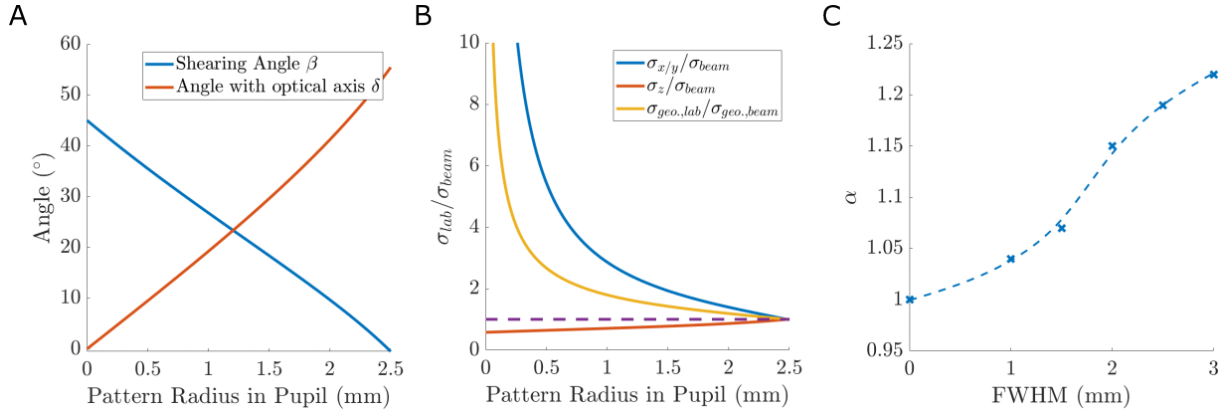


Figure 2.2: **Factors influencing the photon efficiency in a 3D-4Pi MINFLUX measurement:** **A.** Shearing angle β of the beam coordinate system relative to a cartesian system and angle of the individual beams with the optical axis δ as given in Equations 2.22 and 2.21. The angles change approximately linearly with the pattern radius in the bfp. **B.** Ratio of σ in the laboratory system (σ_{lab}) over σ in the beam system (σ_{beam}) and ratio of the 3D detection volume (σ_{geo}) in these two systems as a function of the pattern radius in the bfp. The dashed line is shown as a reference at a ratio of one. For smaller pattern radii, the angle of individual beams with the optical axis decreases, leading to an improved localization precision along the optical axis and a diminished localization precision in the lateral plane. The detection volume becomes the smallest when the beams form a cartesian coordinate system. **C.** Numerical simulation of the increase in the illumination profile's wavelength ($\tilde{\Lambda} := \Lambda\alpha$) as a function of the beams' FWHM. Smaller beams lead to a smaller α which leads to a higher curvature b . The dashed blue line is an interpolation to the values obtained from a numerical simulation of diffracting electromagnetic fields shown as blue crosses.

In addition to the geometry of the beam system, the size of the individual beams also influences the maximally achievable localization precision, as the finite full width at half maximum (FWHM) of the beams leads to an effectively larger illumination profile wavelength $\tilde{\Lambda} := \Lambda\alpha$. The magnitude of α for a given FWHM was calculated numerically¹ and is shown in Figure 2.2. This correction arises due to the coherent summation of differently oriented plane waves in the focus.

In total,

$$\begin{aligned}\sigma_{x/y} &\propto \Lambda(\lambda_0, n) \cdot \alpha(\text{FWHM}) \cdot T_{lat}(r, f, n) \\ \sigma_z &\propto \Lambda(\lambda_0, n) \cdot \alpha(\text{FWHM}) \cdot T_{ax}(r, f, n)\end{aligned}$$

i.e., on the one hand, the photon efficiency depends on sample-dependent parameters such as the excitation wavelength, the focal length of the objectives, and the sample's refractive index. On the other hand, it depends on the beams' FWHM and their placement

¹Numerical simulations were performed with a custom-written *LabView* program, which is provided by Dr. Johann Engelhardt. The program is based on diffraction of electric and magnetic vectors as presented in [65].

in the bfp, which can be optimized to achieve the best localization precision in a 4Pi configuration. In summary, to achieve an optimal photon efficiency, the radius of the beam pattern in the focal plane has to be chosen as large as possible so that T_{lat} and T_{ax} are minimized. Furthermore, the individual beams must be chosen as small as possible to keep α small. Keeping these two requirements in mind, one can optimize a 4Pi setup to obtain the optimal σ_{min} with respect to the curvature b . The background factor a is not considered here, as simulations show it is zero, independent of the specific illumination configuration.

2.3 Comparison between single objective and 4Pi MINFLUX

In the previous chapter, it was shown that a MINFLUX measurement in a 4Pi setup results in an optimal photon efficiency along the axis of measurement (Equation 2.18-2.20). Additionally, it was shown that the beams' FWHM and the beam pattern size influence the achievable photon efficiency in a 3D-4Pi MINFLUX measurement. Here, the theoretically achievable lateral and axial photon efficiency in a 4Pi setup and a single objective setup will be compared. The respective illumination geometries referred to for the remainder of this section are illustrated in Figure 2.3.

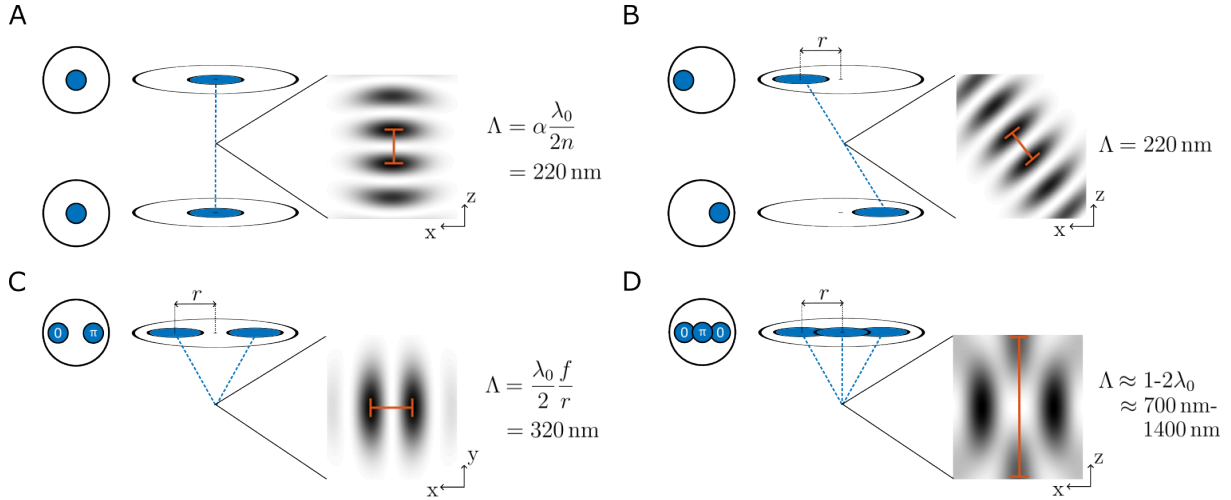


Figure 2.3: **Illumination profiles for 1D-3D localizations in a single-objective and 4Pi setup:** The values for the illumination profile's wavelengths Λ were calculated for $\lambda_0 = 640$ nm, $f = 2$ mm, $n = 1.52$, $r = 2$ mm and FWHM = 1.5 mm. These values are typical for the measurements carried out with the 4Pi setup used in this thesis and for a single objective MINFLUX setup demonstrated in [66] and [63]. The focal length is typical for Leica 100x oil immersion objectives. **A. 1D 4Pi:** Illumination configuration for axial measurements in a 4Pi setup. The wavelength of the illumination profile is 220 nm. **B. 3D 4Pi:** One of the three beam pairs used for performing 3D measurements in a 4Pi setup. As the beams are still counterpropagating, the illumination profile's wavelength is the same as in the 1D case. **C. 2D single objective:** One of the two beam pairs used to perform 2D measurements in a single objective setup. A phase difference of π between the two beams leads to a central intensity minimum with a profile wavelength of 320 nm. **D. 3D single objective:** A third illumination beam in the center of the two outer beams can be used to generate a local intensity minimum along the axial direction in a single objective MINFLUX setup. The wavelength of the illumination profile is on the order of 1-2 λ_0 , depending on the pattern radius. For a radius of 2 mm, it is 720 nm.

In a single objective MINFLUX setup, a 1D local intensity minimum along the x/y-axis is obtained by interference of two beams impinging onto the objective's bfp. The two beams are displaced from the optical axis by a distance of r along the x/y-axis and are polarized perpendicular to the axis of displacement. A local intensity minimum in the center of the focal plane is achieved if the beams have a relative phase of π to each other. This configuration has been reported in [66] and [63] and is sketched in Figure 2.3 C. The achievable photon efficiency only depends on the physical wavelength of the illumination profile, as no coordinate transformation is needed. Furthermore, the influence of the individual beam sizes is not as pronounced as in a 4Pi illumination scheme. Numerical simulations show that $\alpha < 1.05$ for all FWHM. Thus, its influence is neglected for the calculations performed in this section. Plugging Equation 2.21 into Equation 2.19 gives the expression for the wavelength of the lateral illumination profile in a single objective

configuration:

$$\Lambda = \frac{\lambda_0 f}{2 r} \quad (2.26)$$

Here, λ_0 is the wavelength of the beams in vacuum, f is the objective's focal length, and r is the distance of the single beams from the optical axis in the bfp.

It is also possible to generate an axial intensity minimum in a single objective MINFLUX setup. This has already been done with a tophat phase mask [54] and a spatial light modulator [56]. For analogy with the method presented above for lateral illumination intensity synthesis, an axial minimum generated by three interfering beams is presented and discussed. The beam configuration is sketched in Figure 2.3 D. It is similar to the configuration for lateral MINFLUX measurements but features a third beam that propagates along the optical axis. This third beam has the same polarization as the outer two beams. A local intensity minimum is generated if the central beam's phase is shifted by π with respect to the outer beams. There is no analytical expression for the wavelength of the axial illumination profile. However, it is on the order of one λ_0 to several λ_0 depending on the pattern radius and the beam's FWHM.

For the comparison between 3D 4Pi and single objective MINFLUX shown in Figure 2.4, it was assumed that oil immersion objectives with $f = 2$ mm and $n = 1.52$ are used. The calculations were carried out for beams with an FWHM = 1.5 mm, similar to the beam size used in the experiment and for a beam size of FWHM = 0.1 mm, which corresponds to a widefield illumination.

For widefield illumination, a 4Pi setup outperforms a single objective setup irrespective of the chosen pattern radius with respect to lateral photon efficiency, although the influence of the coordinate transformation increases for small pattern radii. For the illumination scheme with an FWHM = 1.5 mm, the lateral photon efficiency achieved with a single objective setup is better than in a 4Pi setup for pattern radii between 1 - 2 mm. This is because of α increasing for larger beams in the 4Pi case. The axial photon efficiency is at least a factor of three better in a 4Pi setup compared to a single objective setup. The 3D photon efficiency is calculated from the photon efficiencies along the individual dimensions according to Equation 2.25. For beams with an FWHM = 1.5 mm, the performance of a 4Pi setup is about 50-60% better than a single objective setup for pattern radii around 2 mm.

It should be noted here that calculations have been performed with an $n = 1.52$, which is the sample medium's refractive index. Therefore, the comparison above is not valid for samples with an index of refraction close to water ($n = 1.33$), which is the case for most biological samples. Given such a sample, one could still use the oil immersion objective in a single objective setup. Therefore, the photon efficiency would not change. At the same time, the illumination profile's wavelength in the 4Pi sample would be extended by a factor

of $\Delta = 1.52/1.33 \approx 1.14$, leading to a performance decrease of 14%. Nevertheless, the photon efficiency obtained in a 4Pi configuration will still outperform the photon efficiency in a single objective setup by at least 35%.

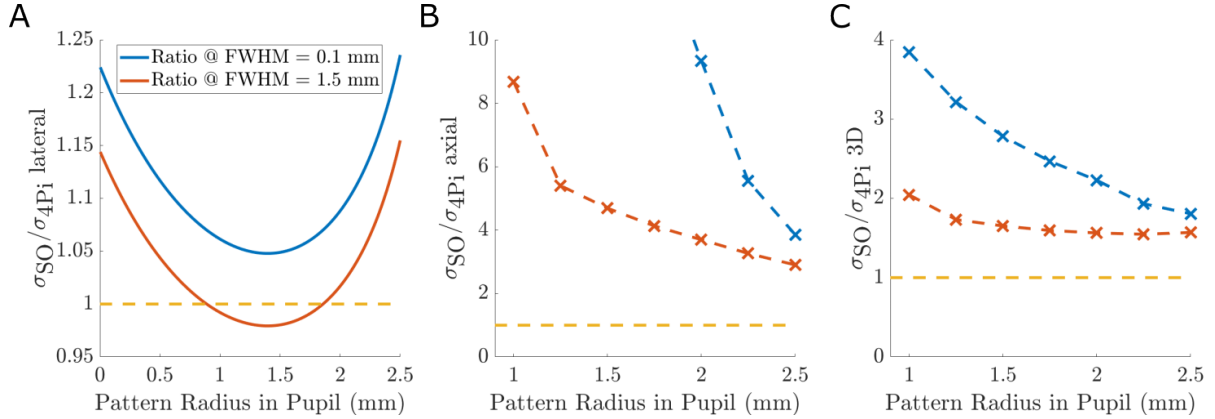


Figure 2.4: Photon efficiency in single objective vs. 4Pi MINFLUX: All ratios presented here were calculated for oil immersion objectives measuring a sample with an index of refraction of $n = 1.52$. Blue curves show the values for beams with an FWHM = 0.1 mm, and red curves show the values for beams with an FWHM = 1.5 mm. The yellow dashed line is introduced at a ratio of one as a guide to the eye. **A.** Ratio of photon efficiency for lateral localizations in a single objective setup and a 4Pi setup as a function of the pattern radius calculated according to Equations 2.19 and 2.26 with corresponding α values. For beams with FWHM = 1.5 mm the two setups have a similar photon efficiency whereas for smaller beams single objective MINFLUX is outperformed by 4Pi MINFLUX. **B.** Numerically calculated ratio of photon efficiency for axial localizations in a single objective setup and a 4Pi setup as a function of the pattern radius. A 4Pi configuration outperforms a single objective configuration by at least a factor of three. **C.** Ratio of photon efficiency for 3D measurements obtained from the calculated and simulated values shown in A and B showing an increase in photon efficiency of about 50% for beams with FWHM = 1.5 mm and pattern radii around 2 mm.

Until this point, for the comparison between single objective and 4Pi MINFLUX, only the curvature b was considered. The second factor influencing the photon efficiency is the offset in the intensity minimum a . It was not discussed because there is no fundamental reason for background sources such as detector dark counts or autofluorescence from within the sample to differ between a single objective and a 4Pi setup. Furthermore, the residual intensity in the minimum is theoretically zero for all illumination schemes presented in this section. Nevertheless, the quality of the intensity minimum obtained in an experiment is never matched with simulations due to residual misalignments and aberrations. The main factors influencing the intensity minimum are the individual beams' polarization, intensity, position, angle, collimation and coherence. These beam parameters have intricate relationships with various parts of the setup. In fact, a 4Pi setup consists of many more components than a single objective setup, including but not limited to a second objective. This aggravates an efficient alignment while also introducing more sources of aberrations.

Therefore, a good intensity minimum is more easily achieved in a single objective setup than in a 4Pi setup. The following chapter will discuss how a good intensity minimum can be achieved in a 4Pi setup with a flexible beam scanning approach despite unfavorable conditions.

Finally, in a 4Pi configuration, twice as many fluorescence photons can be collected than in any single objective setup making it twice as photon efficient concerning emitted photons. This leads to an increase in STP by a factor of $\sqrt{2}$.

Chapter 3

A 3D-4Pi MINFLUX Microscope Designed for Highest Precision

In this chapter, the custom build 3D-4Pi MINFLUX microscope used for the measurements presented in Chapter F is described in detail. It is an extension of the 4Pi MINFLUX system presented in [64], which was developed by Dr. Marcus Held¹. The difference between the 4Pi MINFLUX system presented here, and the previously reported setup is its 3D capability and improved phase scanner module. Here, 3D measurements are enabled by the beam scanning module presented in Section 3.3. The improved phase scanner module is discussed in Section 3.2. An external interferometer measuring the position of the sample was installed to obtain a coordinate transformation from the 4Pi beam system to the laboratory system, as described in Section 3.4. Furthermore, diagnostics tools were added for the control and calibration of the 3D-4Pi PSFs, as outlined in Section 3.6. The localization routine employed with this setup is given in Section 3.5, and the sample preparation is described in Section 3.7.

3.1 Setup

The setup is designed such that isotropic 3D measurements can be performed independently from the excitation wavelength, which can be changed in microseconds by reprogramming the electro- and acousto-optic components. Additionally, care was taken in the design to keep noise contributions to a minimum. Here, noise refers to temperature fluctuations, airflow, vibrations, and electronic noise.

¹Department for Optical Nanoscopy, Max Planck Institute for Medical Research, Germany

Optical Setup

The whole setup is placed on an air-cushioned optical table (I-2000 isolators and RS4000 plate, Newport Spectra-Physics GmbH, Darmstadt, Germany) to decouple it from the vibrations of the building. Furthermore, we build an aluminum frame (30 mm profile system, MayTec Aluminium Systemtechnik GmbH, Olching, Germany) around the setup, which hosts two layers of acoustic foam (Pur Skin, SONATECH GmbH + Co. KG, Ungerhausen, Germany). This is done to shield the setup from airflow and acoustic noise. All lasers are installed on another optical table preventing vibrations from cooling fans and heat transfer to the MINFLUX setup. Also, most of the electronics and the PC are placed on an additional frame coupled to the wall directly above the optical table. This allows for the use of short cables while also decoupling the setup from vibrations. Additionally, single parts of the setup are separated by darkening walls to reduce airflow inside the setup and ensure temperature stability.

The optical setup is depicted in Figure 3.1. The whole setup can be broken down into several modules: A laser module, the beam displacement module, the phase scanner module, the 4Pi interferometer, a diagnostics module, and a detection module.

Laser module The laser module is built on a standalone optical table. The excitation laser light is generated by two single-frequency lasers with a wavelength of 640 nm (Bolero, Cobolt AB, Solda, Sweden) and 560 nm (Jive, Cobolt AB, Solda Sweden). The two wavelengths are combined at a long pass filter (LP, Di03-R561-t1-25x36, IDEX Health & Science LLC, Rochester, US). After that, they pass through an acousto-optic tunable filter (AOTF, PCAOM-VI, Crystal Technology Inc., Palo Alto, USA) which selects the desired wavelength and modulates its intensity. Then, the excitation light is coupled into a polarization-maintaining single-mode fiber (SMF, P3-630PM-FC-10, Thorlabs Inc., Newton, USA) via a fiber collimator (L1, 60FC-4-RGBV11-47, Schäfter + Kirchhoff GmbH, Hamburg, Germany). A half-wave plate ($\lambda/2$, half-wave plate, B Halle, Berlin, Germany) rectified the beam's polarization to the transmission direction of the fiber.

Beam displacement module Before entering the beam displacement module, the excitation light is collimated by a fiber collimator (L1) before passing a half-wave plate ($\lambda/2$, half-wave plate, B Halle, Berlin, Germany) to set the polarization perpendicular to the optical table. After that, a laser line filter (F1, ZET405/488/561/640xv2, AHF Analysentechnik AG, Tübingen, Germany) performs spectral filtering of the beam to get rid of light frequencies which can be generated in the optical fiber due to phase fluctuations. Next, the beam passes through a telescope consisting of two lenses (L2, $f = 60$ mm and L3, $f = 30$ mm). The telescope ensures that the beam pattern generated

in the beam displacement module can be accommodated by the crystal optics in the phase scanner module. A Glen Laser polarizer (GL, GL10-A, Thorlabs Inc., Newton, US) cleans the polarization before the beam enters the pair of 2D acousto-optical deflectors (2D-AODs, AA.DTSXY-400-532(450.650)-011, Pegasus Optik GmbH, Wallenhorst, Germany). The deflectors displace the beam along the two directions perpendicular to the axis of propagation. The working principle of this setup is discussed in detail in Section 3.3.

Phase scanner module A GL cleans the polarization and sets it perpendicular to the optical table. Then, a motorized half-wave plate ($\text{mot}\lambda/2$, half-wave plate, B Halle, Berlin, Germany) mounted on a motorized precision rotation stage PRM1/MZ8, Thorlabs Inc., Newton, US) is used to tilt the polarization by roughly 45° . This ensures equal intensities in the two interferometer beam paths inside the 4Pi interferometer. Subsequently, the beam passes two RTPrubidium titanyl phosphate (RTP) crystals (EOM1 and EOM2, 8x8x25mm custom build, CRISTAL LASER, Messein, France), which are used as phase modulators. A detailed description of this setup follows in Section 3.2.

4Pi interferometer After the phase scanner module, the beam passes through a telescope (L3 and L2). This serves two purposes. First, it enhances the displacement of the beam displacement module by a factor of two. Second, the beam diameter is enhanced by a factor of two, which ensures collimated beams inside the 4Pi interferometer. Behind the telescope, the beam is split into two paths by a polarizing beam splitter cube (PBSC, PBS201, Thorlabs Inc., Newton, US). After the PBSC, both beams pass through identical optical elements. This ensures symmetry between the two beams, which generate an interference pattern between the objectives at the position of the sample. Additional mirror reflections establish a relative beam inversion between the two paths in one of the two paths. Furthermore, one of the mirrors in the lower path can be moved by a tip/tilt piezo scanner (S-316, Physik Instrumente (PI) GmbH & Co. KG, Karlsruhe, Germany) to obtain confocal images through the lower objective. Inside the interferometer, both beams are guided through a GL and a pair of motorized half- and quarter-wave plates ($\text{mot}\lambda/2$ and $\text{mot}\lambda/4$). This ensures complete control over the polarization state of the two beams in the pupil plane of their respective objective. Each interferometer arm hosts a mechanical beam block (mechBB) which is used to change between illumination through one objective and illumination through both objectives. The illumination light and fluorescence light are split at a dichroic beam splitter (DM, ET435/510/595/705, AHF Analysentechnik AG, Tübingen, Germany). Depending on the sample, a pair of oil immersion objectives (HC PL APO 100x/1.44 OIL CORR CS, Leica Microsystems GmbH, Wetzlar, Germany) and a pair of water immersion objectives (Leica HC PL APO STEDwhite 86x/1.20 W motCORR, Leica Microsystems GmbH, Wetzlar, Germany) are used. The lower objective

can be moved in the lateral plane by two piezo motors, whereas the upper objective can be moved along the axial direction with a piezo motor (Piezo LEGS® Linear Twin- C 450N, PiezoMotor Uppsala AB, Uppsala, Sweden). The sample itself is mounted on a custom build sample holder, which is attached to the sample stage (Smarpod 110.45.2-d-sc-149, SmarAct GmbH, Oldenburg, Germany) via magnets. The position of the sample can be tracked in the laboratory system via an external interferometer (Picoscale, SmarAct GmbH, Oldenburg, Germany).

Diagnostics module About one percent of the illumination light passes through the two DMs and is guided into the diagnostics module. The two beams are combined on a beam-splitter cube (BSC, BS010, Thorlabs Inc., Newton, US). On a second BSC, the beams are split into two paths. The first beam passes through a lens (L4, 50mm) and is imaged on a camera (Cam 1, daA2500-14um, Basler AG, Ahrensburg, Germany) in the focal spot of L4. The second beam is directed toward a second camera (Cam 2, acA1920-155um, Basler AG, Ahrensburg, Germany), which is mounted in a plane conjugate to the pupil planes of the two objectives. The spots of the different beams utilized for a three-dimensional MINFLUX measurement are monitored on these cameras to enable the referencing scheme described in Section 3.3.

Detection module The fluorescence light from the two objectives is combined on a BSC. The two output ports of the BSC are each followed by a confocal detection path. Each of the detection paths hosts a motorized pinhole (motPH, MPH16-A, Thorlabs Inc., Newton, US), which has a lens (L7, 75mm) attached to it. One of the detection paths has a lens (L5, 160mm) with a focal length of 160mm, forming a telescope with L7. This detection path consists of a single avalanche photodiode (APD1, SPCM-AQRH-13-FC, Excelitas Technologies, Waltham, MA, USA) with two filters (F2 and F3) in front. The filters ensure the blocking of residual light from the excitation source. They can be exchanged depending on which wavelength is used for the measurement. A lens (L4, 40mm) focuses the fluorescence light onto the APD. The second detection beam path has a lens (L6, 200mm) mounted in front of the motorized pinhole. Behind the pinhole, the detection light is spectrally split into three separate detection paths. The wavelengths around 700 nm are transmitted through the two bandpass filters F4 (FF01-698/70-25, Semrock Inc., Rochester, US). The transmitted light is focused onto APD2 (same model as APD1) by L4. The light which is deflected from F4 is guided toward another pair of bandpass filters F5 (ET600/50m, Chroma Technology Corp, Bellows Falls, US). These filters transmit the wavelengths around 600 nm toward APD2 (same model as APD1). The reflected light from F5 is directed toward a photomultiplier tube (PMT, H9305-03, Hamamatsu Photonics K.K., Hamamatsu City, Japan). This PMT collects the residual laser light transmitted

through the dichroic mirrors. It is used for alignment purposes.

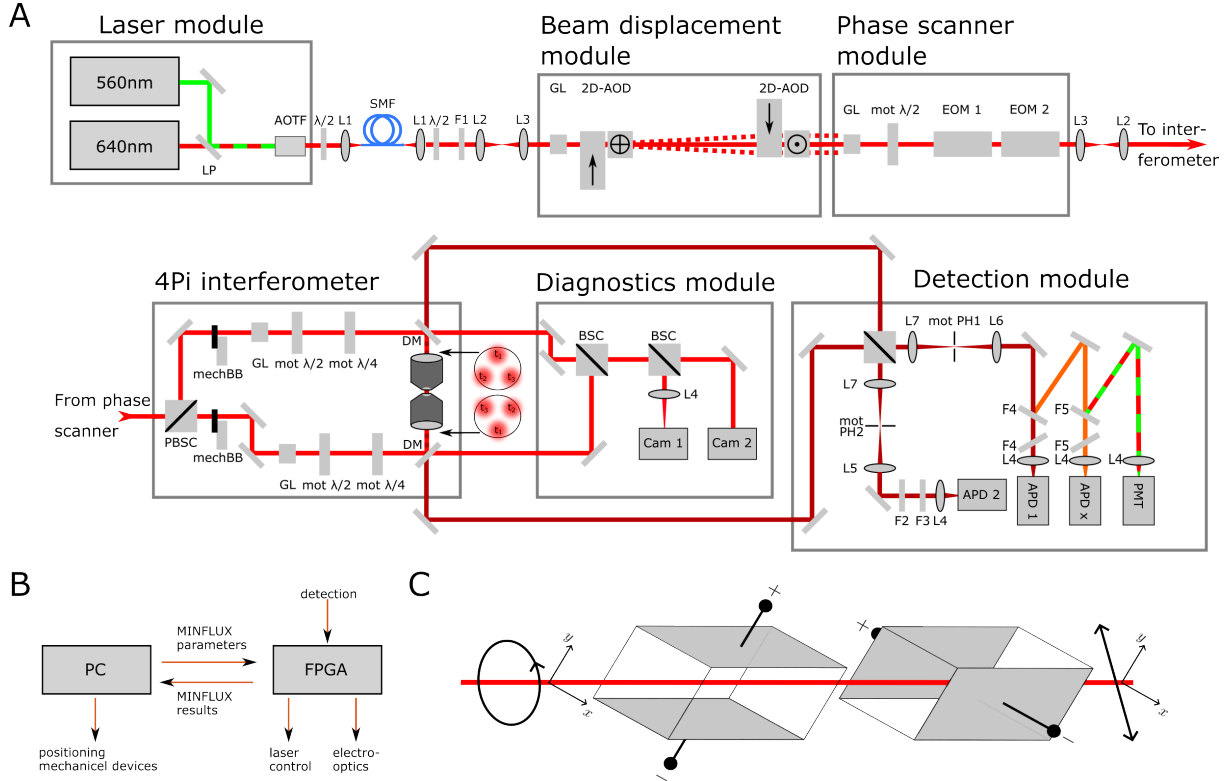


Figure 3.1: **Sketch of the 3D-4Pi MINFLUX setup:** **A.** The entire optical setup of the 3D-4Pi microscope, including all components described in Section 3.1. The beam displacement module is used to address the measurement axis, the phase scanner changes the local intensity minimum position along the beams' axis of propagation, and the beam is split and brought to interference in the 4Pi interferometer. **B.** Structure for communication with electronic devices; fast feedback is guaranteed by using an FPGA. **C.** Detailed sketch of the phase scanner module, which imprints the phase onto the beams' polarization degree of freedom and is designed for maximum temperature stability. Here, the y-axis is perpendicular to the optical table.

Software and Electronics

All electronic devices are controlled with a custom-written *LabView* code running on a PC with Windows 10. The PC communicates with all mechanically moving parts, such as beam shutters, motorized pinholes and λ -plates, the objective motors, and the sample stage. Devices that need to be addressed during a MINFLUX measurement are directly connected to a field-programmable gate array (FPGA), which performs real-time calculations with a frequency of 100 MHz (PCIe-7852, National Instruments, Austin, US). The FPGA is responsible for photon detection, laser controlling, and steering the phase and beam scanner modules. It is used to calculate the position of the emitter from a MINFLUX measurement via Equation 2.5 and adjust the phase to this position in less

than a microsecond. Therefore, the speed of a MINFLUX measurement is only limited by the rise time of the high-voltage amplifiers powering the electro-optic components. After a MINFLUX measurement, data are transferred to the PC. All relevant connections are illustrated in Figure 3.1.

3.2 Phase Scanner

The phase scanner is responsible for shifting the phase of two counterpropagating excitation beams in the 4Pi interferometer. The induced phase shift leads to a shift of the excitation minimum along the axis of propagation of the two interfering beams. In the setup presented in this thesis, this mechanism is employed to perform MINFLUX localizations along the axis of propagation of the beams in the shared focal plane of the two objectives.

Ideally, the phase scanner changes the phase in sub-microseconds; it is stable against temperature fluctuations, it works for multiple wavelengths, and it leaves other beam parameters unaltered. In the remainder of this section, it is shown how these requirements are met with a setup consisting of two RTP crystals, which is used in the 4Pi setup.

Physical Principles

The RTP crystals in the phase scanner are used as electro-optic modulators (EOM). Phase scanning relies on the linear electro-optic effect, also called Pockels effect. The Pockels effect occurs in certain crystals when a direct current (dc) electric field is applied to them. The dc field leads to a redistribution of charges in the crystal lattice, which in turn leads to a change of the crystals index ellipsoid. A thorough derivation of the effect can be found in the book by Yariv & Yeh on pp. 220 [67]. To understand the importance of this effect in the realm of a phase scanner for a MINFLUX microscope, it is sufficient to look at the refractive indices along the two polarization axes of a laser beam passing the crystal. They are given as a function of the applied electric field E .

$$n_{s/p}(E) = n_{s/p} - \frac{1}{2}r_{s/p}n_{s/p}^3E \quad (3.1)$$

Here, $n_{s/p}$ are the indices of refraction of the crystal parallel to the applied electric field and perpendicular to it. They correspond to the refractive indices of the two polarization components of a laser beam going through the crystal. $r_{s/p}$ are the corresponding linear electro-optic coefficients. Therefore, a laser beam propagating through a crystal will experience a dc electric field dependend difference in optical path length between the two polarization dimensions.

This results in a relative phase shift of

$$\Delta\phi(E) = \frac{\pi L E}{\lambda_0} (r_s n_s^3 - r_p n_p^3) \quad (3.2)$$

with the wavelength in vacuum λ_0 and L being the length of the crystal.

The dc electric field over the crystal is given as $E = V/d$ with the applied voltage V and the thickness of the crystal, d . The modulator is characterized by its half-wave voltage. It is the voltage that needs to be applied to result in a phase shift of π :

$$V_\pi = \frac{\lambda_0 d}{L} \frac{1}{r_s n_s^3 - r_p n_p^3} \quad (3.3)$$

Additionally, the relative phase shift is influenced by temperature fluctuations as a change in temperature leads to thermal expansion and a change in the refractive index. The effect of temperature fluctuations can be minimized by using two crystals which are turned 90° to each other along the axis of propagation of the laser beam,

Finally, the phase shift between the two polarization components of the laser light can be separated into two paths by using a polarizing beam splitter.

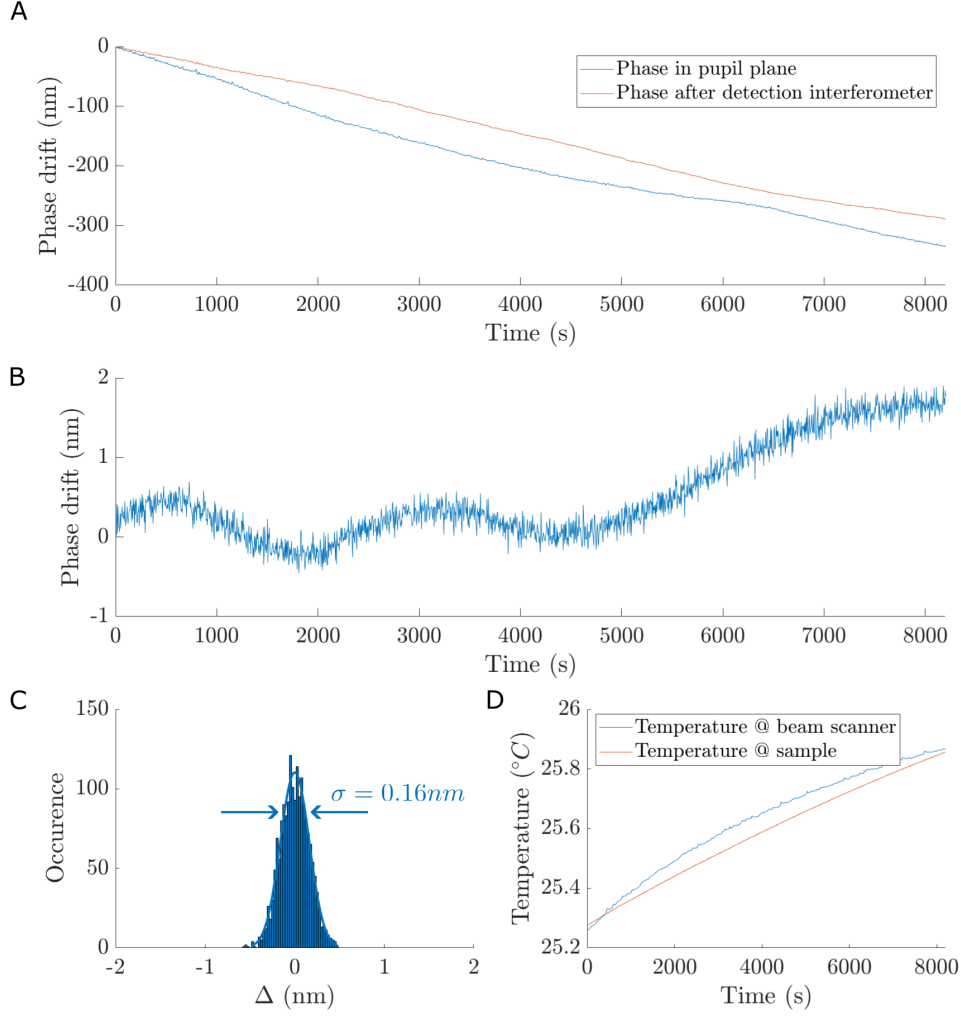


Figure 3.2: **Phase stability measurement:** **A.** Phase drift in the pupil plane of the objective measured on Cam2 and behind the recombining BSC in the detection beam path measured on the PMT for over two hours. **B.** Measurement of the phase drift between the two polarization directions of the laser beam directly behind the phase scanner module performed simultaneously with the measurements in A. The phase drift introduced by the phase scanner is two orders of magnitude smaller than the drift introduced by temperature fluctuations inside the interferometer. **C.** Histogram of phase differences between consecutive measurements of the relative phase directly behind the phase scanner module demonstrating its sub-nm phase stability. **D.** Temperature drift during the measurements presented above. The temperature was measured on one of the two crystals in the beam scanner and at the position of the sample.

Experimental Implementation

A schematic of the phase scanner used in the 3D-4Pi setup is depicted in Figure 3.1. It consists of two 8x8x25mm x-cut RTP crystals, which have the electro-optic coefficients shown in Table B.1. This geometry yields a half-wave voltage of 750 V for 640 nm excitation light and 656 V for 560 nm excitation light according to Equation 3.3. The two crystals are 90° turned to each other to ensure maximal temperature stability. This way,

the thermo-optic coefficients and thermal expansion coefficients of the respective crystal axes cancel out from the temperature-dependent relative phase shift. Values for these coefficients are also given in Table B.1. Differential temperature shifts can lead to more substantial phase fluctuations, which is why the crystals have been placed closely together. For quantifying the stability of the phase scanner module, part of the laser beam was coupled out after the phase scanner and before the PBSC. The laser intensity was observed on a photodiode placed behind a polarizer with polarization axes turned 45° to the crystal axes. The intensity was modulated by continuously applying voltage scans to the phase scanner module. Consecutive phase scans were performed for two hours, and the absolute phase shift was obtained by fitting a $\sin^2$ function to the resulting intensity pattern. Additionally, the phase was measured on Cam2 and on the PMT while all mechanical beam shutters were open. The signal on the PMT gives the phase difference between the two different excitation beam paths from the PBSC up to the DM, plus the phase difference in the two detection beam paths from the DM up to the BSC in the detection module. The phase difference on Cam2 stems only from the excitation beam path up to the DM. Phases from the PMT were extracted the same way as the phases from the photodiode directly behind the phase scanner. The camera signal was taken between voltage scans, Fourier transformed along the axis of intensity modulation, and the phase of the most prominent frequency component was extracted. The results of these measurements are shown in Figure 3.2.

This measurement demonstrates that the phase scanner module's phase drift is negligible compared to other phase drifts in the setup. The relative phase recorded on Cam2 and the PMT are similar, suggesting that the majority of the phase difference is accumulated in the excitation interferometer. The temperature drifted about 0.5° during the whole measurement. Therefore, we could demonstrate the stability of the phase scanner module under varying experimental conditions.

3.3 Beam Scanner

3D measurements are enabled by tilting the measurement axis in rapid succession between individual 1D measurements. The tilt is introduced by addressing different positions in the objective's bfp, which requires a device that can alter the beam path of a laser beam or an on-off switching mechanism between different beams. A beam scanning system was implemented because it is more flexible than on-off switching and requires fewer additional optical components. This not only enables switching between a predetermined set of beams but also allows addressing every position in the objective's bfp. Therefore, the beam scanning approach can partly correct the multitude of residual aberrations introduced through the two objectives and the sample itself. Thus, the beam scanner facilitates the

intricate task of minimizing the intensity minimum mutually for all pairs of illumination beams needed for a 3D measurement. Indeed, the beam scanner is reprogrammed daily to adjust for aberrations caused by the sample.

We implemented the beam scanner with acousto-optic devices as they can be precisely controlled with a radio frequency (RF) signal. Furthermore, they can be switched in microseconds and are usable independent of the excitation wavelength. It should be noted that other implementations for beam scanning systems exist, but they are either too slow (like galvanometric mirrors) or lack a sufficient aperture (like electro-optical deflectors). The physical principles behind AODs and the implementation in the 4Pi setup are discussed below.

Physical Principles

An AOD consists of a crystal and a piezoelectric transducer, generating an RF electric field that produces sound waves propagating through the crystal. This electric field induces an elasto-optic effect which alters the crystals' optical behavior. A full description of this phenomenon lies outside this section's scope but can be read in [68] and [69]. In a nutshell, the sound wave acts as a moving grating to an incoming laser beam. Thus, a laser beam propagating perpendicular to the applied RF field can be deflected according to:

$$\Delta\Theta \approx \frac{\lambda}{v_s} \Delta f \quad (3.4)$$

Here, $\Delta\Theta$ is the deflection angle that is obtained for a change in RF frequency Δf given a laser wavelength λ , and the velocity of sound in the crystal v_s . The equation is only valid under the assumption of small angles Θ . It should be noted here that Θ is not only the deflection angle but also the angle of incidence. Therefore, the acoustic field needs to be divergent, and its limits the maximum achievable deflection.

For generating a beam scanner along one dimension, two AODs need to be placed at a distance d , where the acoustic field in the second AOD propagates subtended to the field in the first one. In the case of equal RF frequencies, a net deflection of zero is obtained, and the position of the outcoming beam is shifted by

$$\Delta x = \tan(\Delta\Theta)d \approx \Delta\Theta d \quad (3.5)$$

where the small angle approximation has been used. Therefore, the beam's position is linearly connected to the RF frequency Δf .

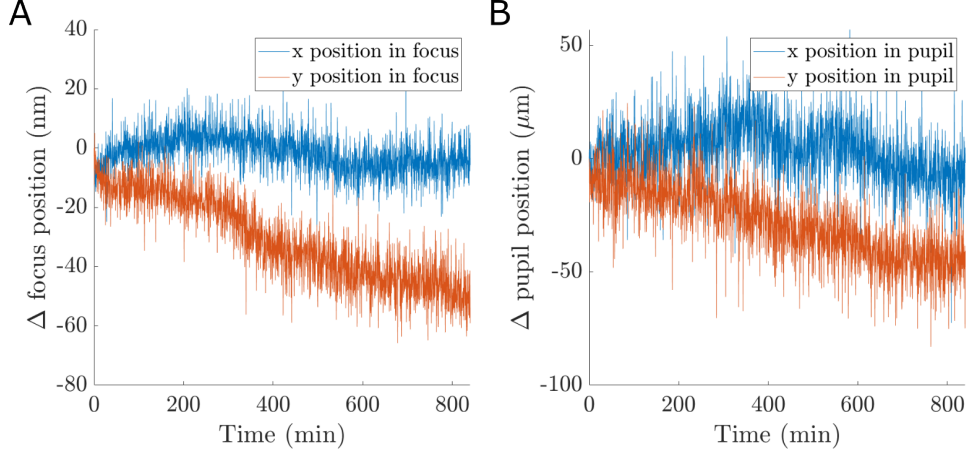


Figure 3.3: **Long-term measurement of beam position:** **A.** Focus position of the excitation beam going through one of the interferometer paths. The position was recorded on Cam1 for over twelve hours and divided by a factor of $f_{L4}/f_{Obj} = 25$ to account for the magnification between the focus of L4 and the focus of the objective. A daily adjustment of the focus position compensates for the long-term drift, which is negligible during the time of a measurement. **B.** Position of the illumination beam on Cam2 corresponding to the position in the objective’s bfp. Again, the drift in pupil position is compensated for by a daily adjustment, but has a negligible effect on a single measurement.

Experimental Implementation

The optical setup consists of two custom-built 2D-AOD modules separated by a distance of $d = 20$ cm. The specifications of the devices are given in Appendix C. Each of the four AODs receives pre-amplified signals from a direct digital synthesizer (DDS, AA Opto Electronic, Orsay, France). DDSs are fed with 32-bit signals, where 8 bits encode the signal amplitude, and 24 bits encode the frequency. Due to the large number of digital outputs, we employ a second FPGA (XC2-XL, Digilent, Pullman, US) running a custom-written VHDL (Very High Speed Integrated Circuit Hardware Description Language) code. The second FPGA receives a series of single-bit signals from the main FPGA and acts as a shift register. Gated by an enable signal from the main FPGA all values on the second FPGA are applied to the DDSs simultaneously. With this configuration, it is possible to change the frequency in steps of 30 Hz in the range of 50-120 MHz. Therefore, according to Equation 3.5, the smallest frequency step leads to a displacement of less than 10 nm. For a frequency step of 10 MHz, a displacement of about 2.8 mm can be achieved, such that the whole aperture of the objectives can be addressed. Nevertheless, the 2D-AOD and the phase scanner are placed between two telescopes, reducing the beam size by a factor of two with respect to the following optics. This is done because the deflection efficiency of the AODs decreases for larger deflection angles.

To test the stability of the beam scanner, we measured the beam position overnight. The results of the drift measurement are summarized in Figure 3.3. The measurement was

performed on the two cameras in the diagnostics module, with Cam1 showing the position in the objective's focus and Cam2 showing the position in the pupil of the objective. Spot locations are extracted from a Gaussian fit to the camera images.

The recorded focus positions resemble the position in the objectives focus up to a magnification factor of $f_{LA}/f_{Obj} = 25$. This factor was already considered for the plot shown in Figure 3.3. Thus, the position in the focus only moves away by about 50 nm during one day. The pupil position was observed to change by up to 50 μm during the measurement period. A difference in pupil position can result in a difference in the pattern radius, which changes the beam coordinate system and, thus, the coordinate transformation. Using the Abbe sine condition and the derivation for the coordinate system given in Appendix A, the resulting difference in lateral position is no more than 4%. In the axial direction, it is less than 2%.

The beam stability measurement demonstrates that changes in beam position over several hours are observable. They are small enough to be neglected during a single measurement but must be monitored and corrected over a longer time. Therefore, a calibration routine employing the diagnostics module has been developed.

Calibration via Diagnostics Module

All parts of the setup are constantly drifting due to environmental influences such as vibrations or changes in temperature. Most of these changes are small enough that they cannot be observed, some are observable on the timescale of several hours, and others change so drastically that their effect can be observed during a single measurement. As demonstrated above, the beam positioning is stable enough not to disturb a single measurement. Nevertheless, changes over longer timescales are observed. Therefore, the beam position is re-adjusted at least once per sample. This adjustment is performed using the beam positions from the upper illumination path recorded on Cam1 and Cam2 and a custom-written *LabView* program.

For each beam, the focus and pupil position are obtained from a Gaussian fit to the camera images. Then, the evaluated positions are compared to the expected positions, which were determined when the setup was adjusted. If the evaluated positions differ from the expected positions, the AOD frequencies are changed according to previously measured calibration curves.

It should be noted here that the AODs provide high flexibility in positioning the beam pattern. Therefore, using the calibration procedure outlined above, they can correct deviations arising due to internal drifts and drifts arising from the downstream optical path.

3.4 3D Coordinate System

The beam scanner can address any position in the objective's back focal plane. As described in Section 2.2, three equidistant positions on a circle centered around the center of the objective's pupil are addressed to perform a 3D localization. A set of three measurements is sufficient to get the 3D coordinate, but it can also be inferred from measurements along more than three axes. Another measurement along the optical axis was performed for some measurements in addition to the three outer beams. Here, it is described how the positions measured along any set of beam axes of the illumination beams are transformed into the laboratory system. The transformation matrix is obtained by performing a MINFLUX measurement of a moving emitter along the desired beam axes while simultaneously determining the position of the sample in the laboratory system.

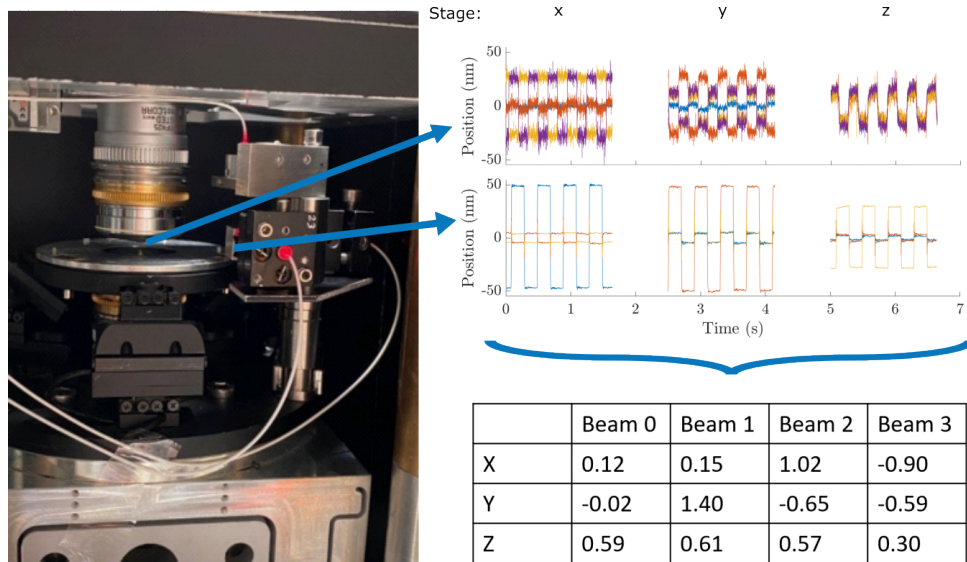


Figure 3.4: **Determining the coordinate transformation:** The picture shows the sample holder in between the two objectives of the 4Pi microscope. A mirrored cube is attached to the right side of the sample holder. The three white optical fibers originate from the PicoScale device. They are attached to the custom-made construct on the right-hand side such that they can measure the position of the sample holder relative to the laboratory system. The positions from a MINFLUX measurement and a measurement of the stage position via the PicoScale during stage movements along the x, y, and z axis are shown in the plots on the right-hand side. The coordinate transformation from the measurement shown in the plots is given in the table on the bottom right. Beam 0 is a beam going through the optical axis, whereas Beam 1-3 are positioned as shown in Figure 2.1. A coordinate transformation from the measurements of Beam 1-3 into the laboratory coordinate system can be obtained from the same calibration measurement.

External Interferometer

The position in the laboratory system is obtained with an external interferometer mounted on a custom-made mount shown in Figure 3.4. This mount is attached to a fixed part

of the stage and hosts three fibers originating from a PicoScale interferometer (SmarAct GmbH, Oldenburg, Germany). Each of the fibers points towards an individual side of a mirrored cube attached to the sample holder. The three fibers can be adjusted such that an infrared laser is reflected back to the fiber, effectively forming a Michelson interferometer with a reference mirror mounted inside the fiber head. This construct is used for finding a transformation from the beam coordinate system to the laboratory coordinate system by monitoring the position of the sample stage during a MINFLUX measurement.

Calibration Routine

For calibrating the coordinate system, the setup is aligned, and a gold nanoparticle (GNP) is brought into focus. Then, a motion of the GNP is induced by moving the stage successively in x, y, and z. Movements are square oscillations with a frequency of 3 Hz and an amplitude of 100 nm in the lateral direction and 50 nm in the axial direction.

While the stage is moving, a MINFLUX measurement along the desired coordinate axes and a measurement of the stage position via the PicoScale interferometer is carried out. The amplitudes from both measurements are extracted with a custom-written MATLAB code. From these amplitudes, the transformations from the stage system to the MINFLUX system $T_{Stage,MF}$ and from the stage system to the laboratory system $T_{Stage,Lab}$ are obtained. Finally, from these two transformations, a direct transformation from the MINFLUX system to the laboratory system is calculated via $(T_{Stage,Lab}^{-1}T_{Stage,MF})^{-1}$.

3.5 MINFLUX Localization Routine

The local minimum needed for a MINFLUX localization is generated in the focal spot of the two objectives by the interference of two counter-propagating waves. MINFLUX steps are carried out in microseconds by applying a voltage to the phase scanner module. A 3D measurement consists of three or more subsequent 1D measurements in which the excitation beams are tilted to each other. Switching between these axes is performed in microseconds by changing the radio frequencies of four acousto-optical deflectors (AOD), which displace the beam laterally at the objective pupil.

For the measurements presented in this thesis, the MINFLUX localization routine consisted of two pre-localization steps with an $L_1 = 120$ nm and an $L_2 = 80$ nm, which were applied subsequently for all measurement axes. The pre-localizations were carried out to bring the intensity minimum of the illumination intensity close to the emitter's position, enabling successive MINFLUX localizations with an $L_3 = 20$ -80 nm depending on the sample. Each pre-localization step was implemented four times to increase the probability of an accurate centering, which failed localizations could prevent.

A single MINFLUX localization along one dimension consisted of a central exposure and two more exposures with the minimum shifted by a distance of $\pm L/2$ along the beam axis. A 3D localization was obtained by performing 1D localizations along all beam axes. For the measurements presented in the following chapter, the beam pairs were arranged on a circle with a radius of 2 mm around the optical axis (as shown in Figure 3.1). An additional pair of beams going through the optical axis was used for some measurements. After each 1D localization, the position of the emitter was calculated in sub-microseconds in the FPGA from the registered photon numbers at the three positions addressed along the beam axis. Then, the relative phase of the illumination beams was adjusted such that the central exposure of the following measurement along this axis was centered around the emitter's position. This calculation was performed according to Equation 2.4 with the additional constraints that the total number of detected photons in this iteration had to exceed a pre-defined background value, the calculated position had to lie within $\pm L/2$, and the curvature (s. Equation 2.13 for $N = 1$) needed to be positive. Otherwise, the position estimate was neglected, and the phase of the illumination profile remained unchanged. The FPGA transferred the data for measured photon counts and estimated position to the host computer, where it was used for further post-processing.

3.6 Alignment Procedure

The drifts of some of the setup's components and the aberrations introduced by them lead to an asymmetry between the two illumination beam paths in a 4Pi setup. This asymmetry manifests in unmatched polarization, intensity, beam position and angle, and collimation between the two beams, which in turn deteriorates the intensity minimum in the mutual focus of the two objectives. This leads to diminished photon efficiency in a MINFLUX measurement. Therefore, the alignment of the setup needs to be monitored and adjusted regularly.

In general, the alignment of the setup can be categorized into three timescales: The long-term alignment, which needs to be checked once per month. A daily alignment, which is performed when a new sample is mounted and a constant optimization for correcting perpetually drifting setup components. The measures that are taken to ensure the correct alignment of the setup are discussed below:

Long-term alignment The polarization and centering of the beam pattern with respect to the optical axis are checked every few weeks. For that, the two objectives are removed from the setup, and the polarization of the beams is checked directly at the objective's pupil with a polarization analyzer (SK010PA, Schäfter + Kirchhoff GmbH, Hamburg, Germany). The motorized $\lambda/2$ and $\lambda/4$ plates in the two illumination beam paths are

adjusted to achieve a matching polarization.

Additionally, the position of the central beam is roughly checked by mounting two pinholes at the position of the two objectives and maximizing the residual intensity behind them in both beam paths. The position is adjusted with the two mirrors preceding each objective. A fine-positioning of the central beam is performed with the objectives re-installed and a sample with a quarter mirror placed in the setup. As the upper objective is fixed in the lateral direction, the position of the beam impinging onto it is adjusted to the optical axis of this objective before the lower objective is adjusted. The adjustment is performed via axial scans of the mirror through the objective's focus while moving the last mirror before the objective to a position where a maximum amount of light is passed back through the fiber, which acts as a pinhole. After that, both objectives are brought to a mutual focus (see 'Daily alignment'). Then, the same procedure adjusts the central beam to the center of the lower objective.

Finally, the positions of the outer beams with respect to the central beam are calculated based on the pattern radius r ; the AODs are adjusted such that the beams fall onto the calculated positions on Cam2 and share a mutual focus on Cam1. This procedure is described in detail in Section 3.3.

Lastly, the central beam needs to be re-centered on the detection pinhole. This is done in analogy to the fine adjustment of the beams in the illumination beam path. Here, the detection pinhole acts as the pinhole, after which the intensity is maximized based on the last two mirrors before the PBSC in each detection beam path.

Daily alignment An alignment of the setup is performed for every sample. First, the AOD frequencies are adjusted such that the positions of the illumination beams are placed at the positions on the cameras in the calibration module, which were determined during the procedure of long-term alignment.

Then, the correction collars of both objectives are adjusted to minimize spherical aberrations. For that, the quarter mirror of the sample is brought into the focus of the respective objective, and axial scans of the mirror are performed while it is illuminated with one of the outer three beams. Simultaneously, the intensity behind the detection pinhole is recorded, and its maximum is maximized by turning the correction collar. For this procedure, it is important to use an outer beam, as it is more sensitive to spherical aberrations. The axial profile of the central beam barely changes for a whole turn of the correction collar. In contrast, changes in the outer beam do become evident already for small rotation angles of the correction collar.

After that, the focal planes of both objectives are aligned with the sample plane via axial stage scans. First, the position of the sample is adjusted to the focus of the lower objective, as this objective cannot be moved along the axial direction. Then, the upper objective's

position is adjusted by placing it to the maximum of an axial scan of the sample by moving the piezo motor attached to it.

Next, the lateral position of the objectives to each other is adjusted. This is done via a sample scan with a GNP emitter in the focal plane for illumination through the two objectives. The difference in the measured position of the GNP for illumination through the upper and lower objective reveals their relative lateral offset. The offset is corrected by moving the lower objective laterally with the attached piezo motors.

Subsequently, the power balance between the upper and lower beam path is adjusted. For that, the central beam's SBR is recorded on a GNP via phase scans and optimized by changing the motorized $\lambda/2$ plate's rotation in front of the interferometer.

After that, the outer beams' focus positions are adjusted to maximize the SBR on a phase scan for each beam. Adjustment of the focus positions is performed by changing the AOD frequencies.

Finally, the procedure described in Section 3.4 is carried out on the GNP to obtain the coordinate transformation.

Constant alignment Some components, which are crucial for the intensity minimum, drift noticeably during the day. The component drifting the most is the sample itself. An axial sample scan adjusts its axial position every few minutes using the back-reflex from the sample surface.

Next, approximately once every hour the position of the two objectives relative to each other is checked on a GNP and adjusted according to the procedure described above.

3.7 Specifics of the 4Pi Sample Preparation

All 4Pi samples consist of one 18 mm, and one 15 mm diameter coverslip (170 μ m, No 1.5H, Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) sandwiched together and sealed with either nail polish (RIVAL loves me Care Power Hardener, Dirk Rossmann GmbH) or two-component glue (2-K-Epoxidkleber Sofortfest, UHU, Bühl, Germany). Before using a coverslip for sample preparation, its thickness is checked to be within $\pm 3\mu$ m of the mean thickness of 170 μ m. This step is taken to ensure equal beam paths for the lower and upper illumination beams. The coverslip sandwich is mounted on an aluminum ring manufactured in our mechanical workshop to fit precisely on the sample stage. All coverslips are washed in acetone and ultrapure water and subsequently plasma cleaned (TePla 300 Semi-Auto Plasma Processor) for 5 minutes under 0.5mbar of O_2 at a power of 200W to remove contaminants from the surface.

The sample is mounted on the upper side of the lower coverslip, with a diameter of 18 mm.

This coverslip has an additional aluminum quarter mirror printed onto its upper side for creating a reflex from the illumination laser, which is used to check the two objectives for spherical aberrations and enables minimizing the aberrations via the objectives' correction collars.

Additional, residual aberrations predominantly arise in the upper illumination path from non-perfectly index-matched mounting media. To keep this effect as low as possible, the thickness of the samples is adjusted to below $20\mu\text{m}$ if no index-matching can be achieved. For each sample, gold nanoparticles (GNP-bt, 40nm Biotin Gold Nanoparticles, Cyto-diagnostics Inc, Burlington, Canada) are immobilized on the surface to facilitate the alignment procedure of the 4Pi MINFLUX system. They are used because they show a high emission rate into the fluorescence channel while keeping this rate for an extended time.

Surface functionalization

The protocol for surface functionalization was developed by Dr. Lukas Scheiderer² from our group. It consists of coating the surface with PLL-PEG-bt (PLL: Poly-L-Lysin, PEG: Polyethylene Glycol, bt: biotin) and subsequently adding NVD (Neutravidin). PLL-PEG binds to the glass surface to prevent the sample from sticking. The biotin is used to functionalize the coverslip so the sample can attach to it. NVD binds to some of the biotin binding sites to thin out the available sites for the subsequently added sample.

The whole procedure consists of applying $50\mu\text{L}$ of PLL-PEG-bt (0.2mg/mL) with 1%Vol Tween(P20) for 15min followed by $50\mu\text{L}$ of NVD (1mg/mL diluted 1:100 in PBS) for 5min. In between steps, the coverslip is washed with 1 ml of phosphate buffered saline (PBS) and dried under nitrogen flow.

²Department for Optical Nanoscopy, Max Planck Institute for Medical Research, Germany

Chapter 4

3D Single Emitter Localization Measurements

The 3D-4Pi MINFLUX setup described in Chapter 3 was characterized by a measurement of single immobilized ATTO647N molecules and on a stage-driven GNP for the achievable STP and photon efficiency. Furthermore, the setup was used to study the stepping behavior of DNA nanorobots and the diffusion of single emitters in a sugar solution, demonstrating its applicability to biological and physical studies of 3D movements on the nanoscale.

4.1 Highest Single Molecule Photon Efficiency

The 3D single-molecule photon efficiency was established on surface-immobilized ATTO647N fluorescent molecules. These fluorophores were chosen as a test system as they are known for their high photostability and brightness.

Sample Preparation

The ATTO647N single-molecule samples were prepared by plasma cleaning and functionalizing coverslips according to Section 3.7. The coverslip was incubated with 50 μ L of a mixture of Atto647N-bt and GNP-bt for 5min. The GNP-bt were diluted 1:100 from stock in PBS, and ATTO647N-bt was added to the mixture in a dilution of 100 pM. After 5min of incubation, the coverslip was washed with 1 ml of PBS. The mounting medium consisted of PBS with 1% VectaCell (CB-1000-2, Vector Laboratories Inc., Burlingame, USA) added to it. VectaCell contains Trolox, which reduces the formation of reactive oxygen species.

Data Acquisition and Analysis

The density of ATTO647N fluorophores was adjusted such that about 3-4 of them occupy a region of $5 \times 5 \mu\text{m}$ on the coverslip. A GNP was used to adjust the setup and sample, as explained in Section 3.6. ATTO647N molecules were pre-localized by performing overview scans with the piezo scanner. An identified emitter was placed inside the detection volume of the microscope by shifting the stage accordingly. Subsequently, a MINFLUX tracking procedure with an $L = 40 \text{ nm}$ and a measurement time of $925 \mu\text{s}$ per axis was carried out for the three beams of the 3D 4Pi MINFLUX pattern and the central beam. The data were analyzed with a custom-written MATLAB script employing the FCE outlined in Section 2.1. Positions in the laboratory system were computed from the positions obtained along the outer three beam axes only. Measurements were carried out using water objectives (Leica HC PL APO STEDwhite 86x/1.20 W motCORR, Leica Microsystems GmbH, Wetzlar, Germany) with an NA of 1.20.

Results

In total, 17 traces of individual ATTO647N molecules were taken. Figure 4.2 shows one of the measured traces. We observe that the localization precision between individual axes differs by up to 55%, which is due to different measured photon numbers and SBRs along these axes. The localization precision in the laboratory coordinate system along the axial direction is 2.5-2.9 times higher than the corresponding lateral localization precision. This is because of the uneven localization precision along the beam axes and the skewness of the beam coordinate system.

The σ_L of all measurements are plotted in Figure 4.1. We observe a higher σ_L in the beam system compared to the laboratory system owing to the skewness of the beam coordinate axes. Most importantly, the setup demonstrates the highest 3D photon efficiency achieved to date on a MINFLUX setup with $\sigma_L = 22.7 \text{ nm}$ along the beam axes and $\sigma_L = 31.3 \text{ nm}$ along the laboratory axes. A comparison to previously published MINFLUX measurements and other superresolution modalities is shown in Table 4.1.

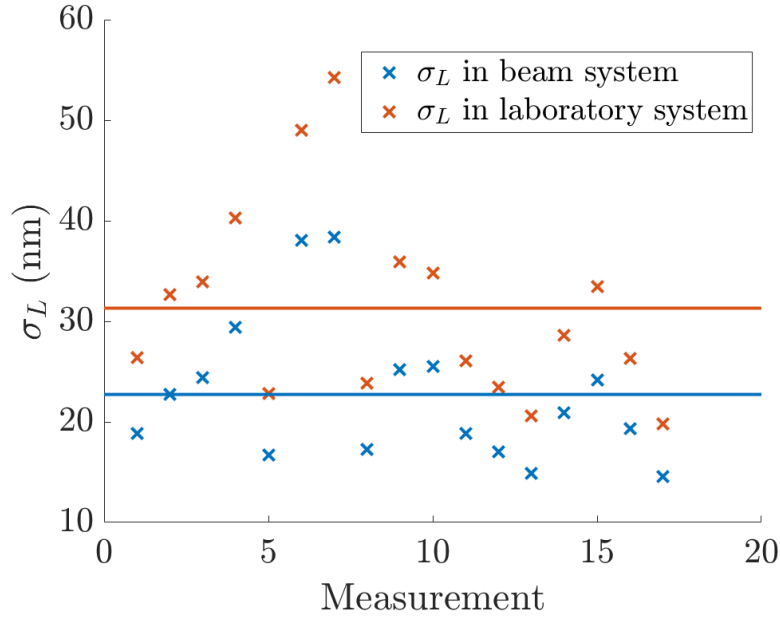


Figure 4.1: **Photon efficiency on ATTO647N** 3D photon efficiency evaluated for the outer three axes of the beam coordinate system for a set of 17 measurements on single immobilized ATTO647N molecules. For blue crosses, the 3D photon efficiency was calculated as a geometric mean (according to Equation 2.25) along the three axes of the beam system. For orange crosses, it was calculated as a geometric mean along the three axes of the laboratory system. Horizontal lines represent the mean value of the photon efficiency, which is 22.7 nm in the beam system and 31.3 nm in the laboratory system, demonstrating a better photon efficiency than any single-objective 3D-MINFLUX measurement.

Method	Publication	σ_{geo} (nm)	N/3	σ_L/σ_{PSF} (nm)	STP ($10^{-12}\text{m}\sqrt{s}$)
4Pi STORM	[30]	3.0	2670	157	287
STORM (4Pi Quantum Cramer-Rao Bound)	[70]	–	–	79	–
isoSTED	[29]	–	–	40-45	–
MINFLUX (January 2020)	[54]	2.4	3330	138	–
MINFLUX (August 2020)	[55]	5.3	670	137	–
MINFLUX (March 2021)	[56]	2.0	400	40	–
MINFLUX Tracking (December 2021)	[62]	8.8	47	60	161
4Pi MINFLUX on ATTO647N	4.1	4.1	62	31.3	214
4Pi MINFLUX on ATTO647N in beam system	4.1	3.0	62	22.7	157
4Pi MINFLUX on GNP	4.2	0.47	442	9.8	8.3
4Pi MINFLUX on Nanorobots	4.3	4.3	32	24.2	227

Table 4.1: **Photon efficiency and STP of selected 3D measurements:** The photon efficiency and STP for the measurements presented in this thesis, together with all previous 3D MINFLUX measurements and selected values for other superresolution techniques, are shown. The measurements presented in this thesis show the highest photon efficiency and the highest STP for the GNP measurement. Furthermore, the single-molecule measurements presented in this thesis show the highest photon efficiency among all 3D superresolution techniques. STP for single-molecule measurements is similar for different measurement modalities but relies strongly on the emission rate of the observed fluorophore.

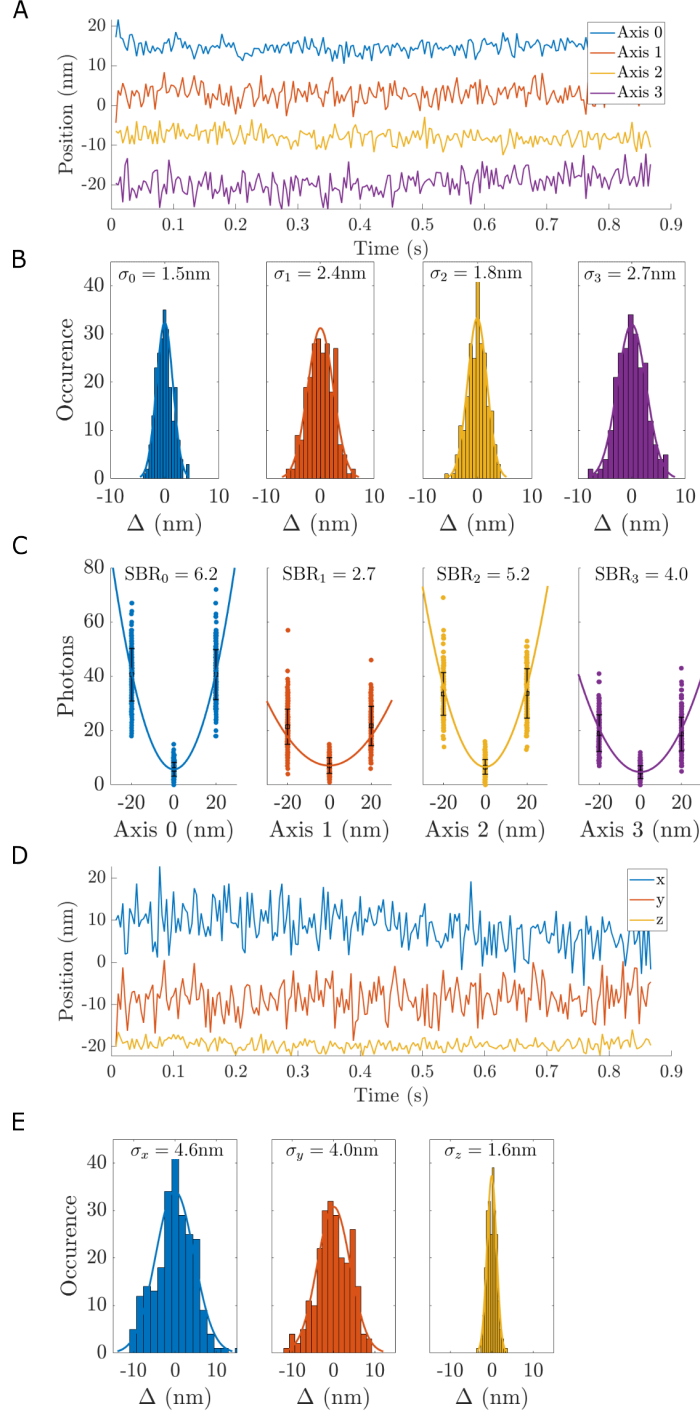


Figure 4.2: **ATTO647N trace and statistics @ $L = 40$ nm** **A.** ATTO647N trace measured along the optical axis (Axis 0) and the three outer axes spanning the 3D-4Pi coordinate system. **B.** Histograms of position differences between consecutive measurements obtained from the traces shown in A. **C.** Corresponding photon distributions and SBR along the four measured axes. **D.** Position of the emitter in the laboratory coordinate system obtained from a coordinate transformation of Axes 1-3. **E.** Histograms of position differences between consecutive measurements obtained from the traces shown in D, giving the localization precision in the laboratory coordinate system.

4.2 Best Spatio-Temporal Precision on a Gold-Nano-Particle

A GNP was used as an emitter for a 3D measurement to demonstrate the setup's calibration and its short-term stability. The GNP was chosen as an emitter as it gives a steady and robust signal in the fluorescence channel. Therefore, it was possible to determine the stability of the setup unaffected by any fluorophore imperfections such as low fluorescence rate, blinking, and bleaching.

Before performing the measurement, the setup was adjusted on another GNP according to the procedure outlined in Section 3.6. The GNP was pre-localized in the lateral direction by performing an overview scan with the piezo scanner. An axial pre-localization was accomplished with a z-scan of the stage. A MINFLUX tracking procedure with an $L = 20$ nm and a measurement time of $115 \mu\text{s}$ per axis along the outer three beam axes was carried out. During the measurement, the GNP was moved along a Lissajous figure with the help of the stage. This movement was additionally monitored with the external interferometer to check the calibration of the coordinate transformation outlined in Section 3.4. The data were analyzed with a custom-written MATLAB script employing the FCE outlined in Section 3.5. Measurements were carried out using oil objectives (HC PL APO 100x/1.44 OIL CORR CS, Leica Microsystems GmbH, Wetzlar, Germany) with an NA of 1.44.

Sample Preparation

Plasma cleaned and functionalized (according to Section 3.7) coverslips were used to prepare GNP samples. The coverslip was incubated with $50 \mu\text{L}$ of a mixture of PBS with GNP-bt diluted 1:100 from stock for 5min. After 5min of incubation, the coverslip was washed with 1ml of PBS. The mounting medium consisted of 2,2'-thiodiethanol (TDE).

Results

Calibration of the setup was inspected on the trajectory of a GNP which was invoked by a stage movement. The recorded movement is shown in Figure 4A. The amplitudes of the injected Lissajous pattern were determined to be $9.7 \text{ nm}/9.7 \text{ nm}/12.9 \text{ nm}$ in x, y, and z via a MINFLUX measurement on the GNP. They were simultaneously measured with an external interferometer monitoring the position of the stage. The amplitude of the stage movement was measured to be $10.9 \text{ nm}/9.8 \text{ nm}/11.3 \text{ nm}$ in xyz. Therefore, the calibration of the beam axes in the laboratory system is exact up to a deviation of at most 15 %. The residual error stems from deviations of the stage movement at the position of the external interferometer compared to the position of the MINFLUX measurement.

Furthermore, at a detection rate of 2.9 kHz for a point in 3D space and a localization precision of $5.2 \text{ \AA}/5.2 \text{ \AA}/3.3 \text{ \AA}$ in xyz, the measurement yields an STP of $8.3 \times 10^{-12} \text{ m}\sqrt{\text{s}}$ for a 3D localization. The values for the localization precision were computed as the standard deviation of consecutive measurements. Therefore, they reflect the short-term stability of the setup. As can be seen in Figure 4.3, the long-term stability is affected by 50 Hz electronic noise, which differs from measurement to measurement, and is an effect of the open construction of the setup, which an improved electric shielding can eliminate. The results of the measurement are summarized in Table 4.1. The table shows that this measurement demonstrates the highest STP and the highest photon efficiency among any 3D MINFLUX measurements. Furthermore, the capabilities of the MINFLUX machine unaffected by fluorophore imperfections have been demonstrated.

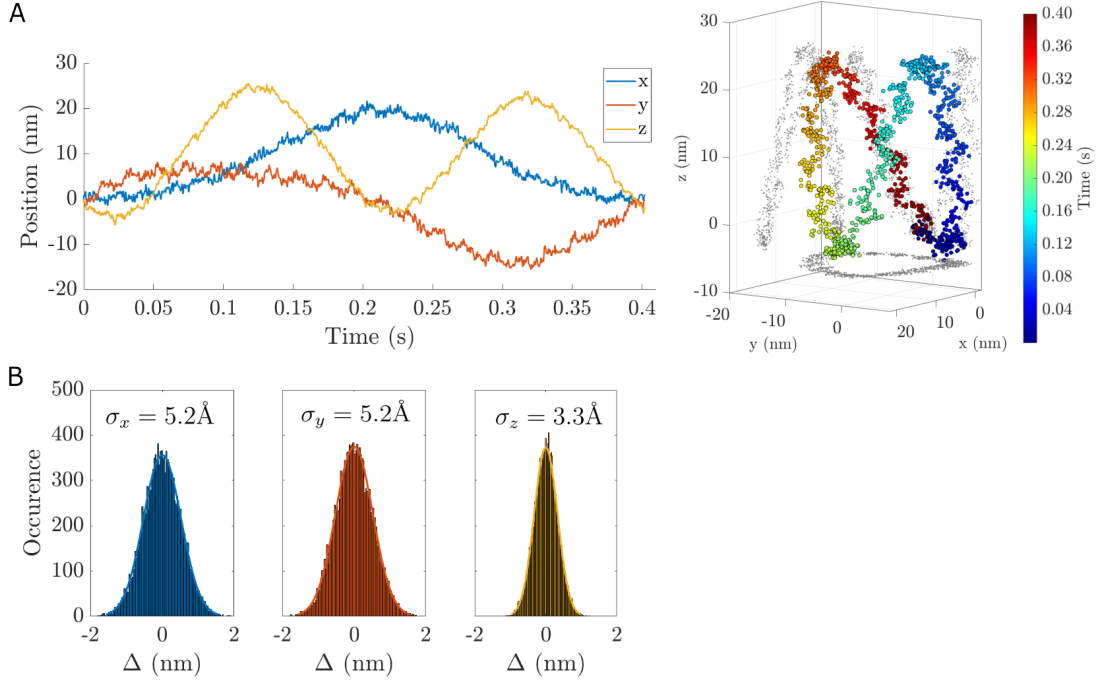


Figure 4.3: **High-precision trace on GNP A.** (Left) Trajectories of the stage-driven GNP along the laboratory coordinate system's x, y, and z-axis. (Right) 3D illustration of the measured trajectory. **B.** Histograms of position differences between consecutive measurements obtained from the traces shown in A demonstrating short-term sub-nanometer localization precision at a 3D detection rate of 2.9 kHz. Over longer periods, 50 Hz electronic noise can be seen on the trace, which is caused by the setup's open construction and can be eliminated by improved electric shielding. The precisions along the three axes give a 3D precision of $\sigma_{geo} = 4.7 \text{ \AA}$.

4.3 Investigations of the Stepping Behavior of DNA Nanorobots

DNA Nanorobots are used as a test system for demonstrating the tracking capabilities of the setup with a biotechnological sample. This investigation aims at showing that the setup can reproduce previous results acquired in 2D MINFLUX measurements while also giving new insights into the 3D movement of the sample.

The sample design was done in close collaboration with Namita Sarraf¹, Prof. Dr. Lulu Qian¹, Prof. Dr. Erik Winfree¹ and Dr. Jessica Matthias² and is based on Anupama J. Thubagere et al. [71].

The Nanorobots are single-stranded DNA (ssDNA) strands that can bind complementary ssDNA poles sticking attached to a base plate (DNA origami). The Nanorobot is made up of two 'feet' (each containing an identical sequence of 6 nucleotides) connected by a 'leg' (15 nucleotides). The ssDNA poles form a track on the base plate surface and are complementary to the Nanorobot's leg (leg*) and to one of the two feet (foot*). The foot* of 'Track 1' sits at the pole's bottom (close to the base plate surface), while the foot* of 'Track 2' sits at the pole's top (distant from the base plate surface). The Nanorobot can 'walk' randomly between the poles without the need to be fueled. The mechanism is based on the interactions between complementary ssDNA strands and reversible strand displacement reactions (Figure 4.4). While attached to a pole, the Nanorobot has its lower or upper foot free to bind to the next track position. Thus, the robot always moves from a Track 1 pole to a Track 2 pole. The movement is initiated by competing binding forces of adjacent track positions, leading to branch migration of the complementary leg* until the whole leg is attached to the next track position. Then, the foot also disassociates from the previous track position.

For tracking the stepping behavior of the Nanorobots, an Atto647N single fluorophore was attached to one of the two feet of the Nanorobot. The baseplate was immobilized on the coverslip via a biotin/neutravidin coupling.

Due to the geometry of the baseplate, step sizes of 6 nm, 10.4 nm and 16 nm in the lateral direction were expected. Furthermore, the sequence identity of the Nanorobot's feet hints at possible axial steps with a step size equal to the length of one leg and one foot of the Nanorobot. This movement corresponds to an axial displacement of 12.2 nm.

¹Bioengineering, California Institute of Technology, Pasadena, CA 91125, USA

²Department for Optical Nanoscopy, Max Planck Institute for Medical Research, Heidelberg, Germany

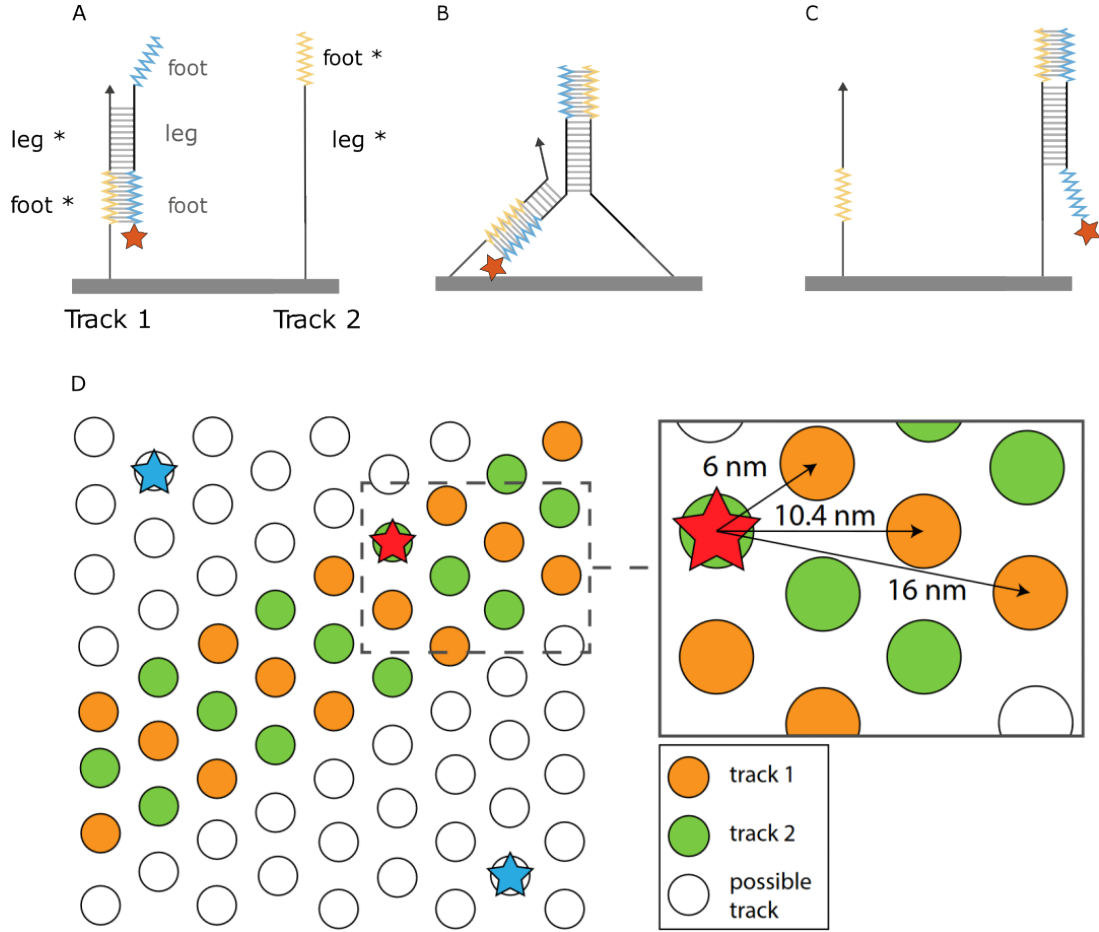


Figure 4.4: **Nanorobot walking mechanism and sample geometry** **A.** The Nanorobot is attached to a pole of Track 1 with its upper foot unbound. **B.** The upper foot of the Nanorobot and parts of the leg are attached to a neighboring pole from Track 2. **C.** The Nanorobot is only attached to a pole of Track 2, and its lower foot is free to bind to another pole of Track 1. **D.** The baseplate is composed of possible pole positions arranged in a honeycomb pattern. Only three neighboring rows of positions are occupied by the poles of Track 1 and Track 2. Different tracks are color-coded and show possible step sizes of 6 nm, 10.4 nm, and 16 nm. Two Alexa488 fluorophores are attached to opposing sides of the track and are shown here in blue. The figure is adapted from [66].

Sample Preparation

We ordered the DNA strands from Integrated DNA Technologies, Coralville, US. The base plate was folded at our institute by Dr. Jessica Matthias³, who also helped with sample preparation. Coverslips were functionalized and plasma cleaned according to Section 3.7. After thoroughly washing with PBS, 50 μ l of 60 pM baseplate solution in 1x TAE buffer / 20 mM MgCl₂ (TAE/MgCl₂) were incubated for 5 min. The MgCl₂ was added to compensate for the repulsion of the negative charges of the DNA backbone. After that, coverslips were washed with 1 ml of TAE/MgCl₂. In the next step, the coverslips were

³Department for Optical Nanoscopy, Max Planck Institute for Medical Research, Heidelberg, Germany

incubated with 50 μl of a 20 pM DNA Nanorobot / 0.1% GNP-bt solution in TAE/MgCl₂. Subsequently, the coverslips were washed with 1 ml of TAE/MgCl₂. To construct a flow chamber, two small stripes of Scotch tape (3M, St. Paul, US) were attached at two opposite outer edges of one side of a 15 mm coverslip. This coverslip was then sandwiched with the sample-coverslip such that the Scotch tape acted as a spacer between the two coverslips. The buffer, a solution of TAE/MgCl₂ containing 1% VectaCell (CB-1000-2, Vector Laboratories Inc., Burlingame, USA), was added subsequently. Due to the construction with the Scotch tape acting as a spacer between the two coverslips it was possible to exchange the buffer when needed.

Simulation and Analysis

A step detection analysis was developed and applied to the data to quantify the results of the tracking measurements on the DNA Nanorobots. The parameters of this analysis were tuned to reproduce the ground truth of a simulation of the Nanorobot stepping behavior.

Simulation Simulations were carried out with a custom-written MATLAB code. For the simulations, the Nanorobot track was constructed according to the scheme shown in Figure 4.4. The base plate could be freely placed in 3D space, and a starting position for the Nanorobot was randomly assigned to one of the poles. The Nanorobot could move randomly from a pole of Track 1 (Track 2) to a pole of Track 2 (Track 1) but would never occupy a position between poles. The movement was only restricted by a decay function, which depended on the distance of the current pole to the next pole. A stepping probability factor could tune the average temporal step length.

Artificially generated Nanorobot trajectories were then subjected to a simulated 3D-4Pi-MINFLUX measurement by transforming the simulated positions into the beam coordinate system and applying a global drift and uncertainty due to photon noise. Subsequently, the trajectories were transformed back to the laboratory system, in which they were used to test the step detection analysis.

3D Step Detection For detecting steps in 1D data, a custom-written MATLAB code was used. The core of this code is the MATLAB function *ischange*. This function iteratively searches for abrupt changes in data by minimizing the objective function [72]

$$\sum_{i=1}^{m+1} \left[C \left(y_{(\tau_{i-1}+1):\tau_i} \right) \right] + \beta m. \quad (4.1)$$

Here, y is the time series under investigation, m is the number of changepoints that occur at positions τ_i , C is a cost function for a segment, and β is a penalty factor. For detecting

abrupt spatial displacements in the data, the cost function $C(y') = N\text{var}(y')$, in which N is the number of data points in a segment y' , is used. This function is minimized if a segment is approximated well by its mean. The only free parameter of the minimization is the penalty factor β . It was shown previously [63] that a reasonable estimate for the penalty factor is $k \cdot \text{var}(y)$, with k chosen big enough such that overfitting of the data is minimized.

The method described above was used to separately detect steps for the trajectories along x , y , and z . In the next step, the steps along these axes were merged to give steps in 3D space. For this, the time points of all steps were investigated for the number of data points between them. The latter step was removed if two steps were detected at consecutive time points along two different axes.

Optimization of detection algorithm The simulation outlined above was used to test the 3D step detection algorithm and optimize its parameters to reproduce the ground truth. A Nanorobot movement with a step size of 6 nm was used to determine a suitable value for the penalty parameter k . For this simulation, step durations and photon noise were chosen, similar to values observed in actual measurements. The results of the simulations are shown in Figure 4.5. The decay parameter k was chosen such that the number of detected steps was smaller than the number of simulated steps, and the median step size was not significantly larger than the simulated step size. For a decay parameter $k = 30$, 75% of steps were detected, and the median step size was found to be 6.1 nm, being close to the ground truth of 6 nm. Lowering k resulted in overfitting, indicated by a smaller median step size and an increasing number of detected steps. In comparison, a larger value for k led to a larger median step size and a larger number of missed steps. Therefore, k was set to 30 to evaluate actual measurements.

Furthermore, a simulation was carried out in the absence of steps, but in the presence of drift, which was comparable to the drift in the setup (approx. 1 nm/s) to see if false positives were generated and how they could be distinguished from real steps. This simulation showed that 98% of false positives were smaller than 3 nm. Therefore, steps smaller than 3 nm were filtered out in post-processing.

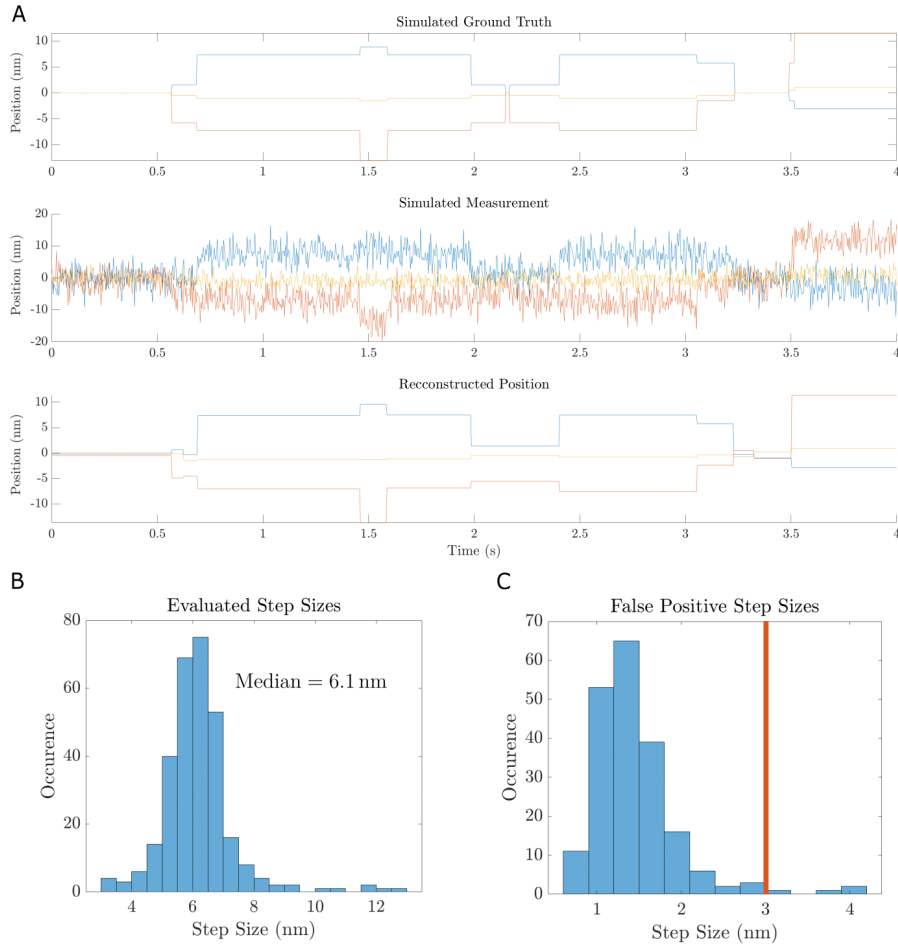


Figure 4.5: **Simulations for Nanorobot stepping** **A.** Time traces for the simulated ground truth of a Nanorobot with a step size of 6 nm, the simulated output position after a MINFLUX measurement with a noise of $\sigma = 2$ nm along the measurement axes and the reconstructed position after a 3D step analysis with the parameters outlined in this section. **B.** Distribution of reconstructed step sizes from the simulation shown above for a simulated duration of 100 s with a median of 6.1 nm, demonstrating the accuracy of the analysis routine. **C.** Distribution of reconstructed step sizes from a simulation without Nanorobot movement but a drift of 1 nm/s indicating the distribution of false positives. 98% of false positives are smaller than 3 nm, which is why steps with a size of less than 3 nm are discarded.

Measurement and Results

The concentration of Alexa488 fluorophores was checked to ensure a sparse distribution of DNA origami baseplates on the coverslip. Furthermore, the concentration of the Nanorobots was kept low compared to the baseplate concentration to keep the occupancy per base plate below one. An overview scan with the piezo scanner was performed to find Nanorobot positions. An identified emitter was placed inside the detection volume of the microscope by shifting the stage accordingly. Subsequently, a MINFLUX tracking

procedure with an $L = 40$ nm along four beam axes and a measurement time of $925\text{ }\mu\text{s}$ per axis was carried out.

An example trace and the corresponding step detection fit are depicted in Figure 4.6A. The wide spread in durations between steps indicates the stochastic behavior of the stepping mechanism. Figure 4.6B-D shows the resulting step size distribution in 3D space, projected onto the z-axis and the lateral plane for the whole set of Nanorobot traces measured on the 4Pi setup. The median step sizes have been evaluated to 10.1 nm, 2.7 nm and 9.3 nm, respectively. The lateral distribution shows some step sizes larger than the maximally expected step size of 16 nm, which is an artifact attributed to underfitting, which can already be observed in the simulated data in Figure 4.5 B. This underfitting can result in step sizes up to twice as large as the maximum step size. It becomes evident that the difference between lateral step size and 3D step size is small compared to the median axial step size indicating that an axial displacement rather occurs together with a lateral displacement. This might happen when the Nanorobot undergoes a step from one track to the other while also flipping the axial position of its two feet and results in an axial step size of 12.2 nm. The axial step size distribution also shows many steps smaller than 12.2 nm. These steps can arise from the DNA origami not lying flat on the coverslip surface. Other causes for deviations of observed behavior from expected behavior are imperfections of the DNA origami baseplate and flexibility of the ssDNA poles, which have a persistence length of only 2 nm [73]. Nevertheless, the observed 3D median step size lies within the range of expected step sizes from the DNA origami geometry.

For a further evaluation of setup performance concerning its accuracy, the data found with the step finding algorithm are compared to previously measured tracks of Nanorobots, which were obtained on a 2D MINFLUX microscope [66] by Dr. Tobias Engelhardt⁴. For this purpose, the 2D MINFLUX data were evaluated using the step-finding algorithm with the same parameters used for the 3D data analysis. The result of this analysis is shown in Figure 4.6 with a median step size of 8.4 nm. Thus, the accuracy of the 3D-4Pi setup and the 2D setup is within 10% of each other. Deviations between the two measurements can also arise due to differences in sample preparation and measurement conditions independent of the setup. An example for the former is salt concentration, which influences the compactness of the DNA origami folding. An example for the latter is temperature, which is the main driving factor in stochastic processes.

⁴Department for Optical Nanoscopy, Max Planck Institute for Medical Research, Heidelberg, Germany

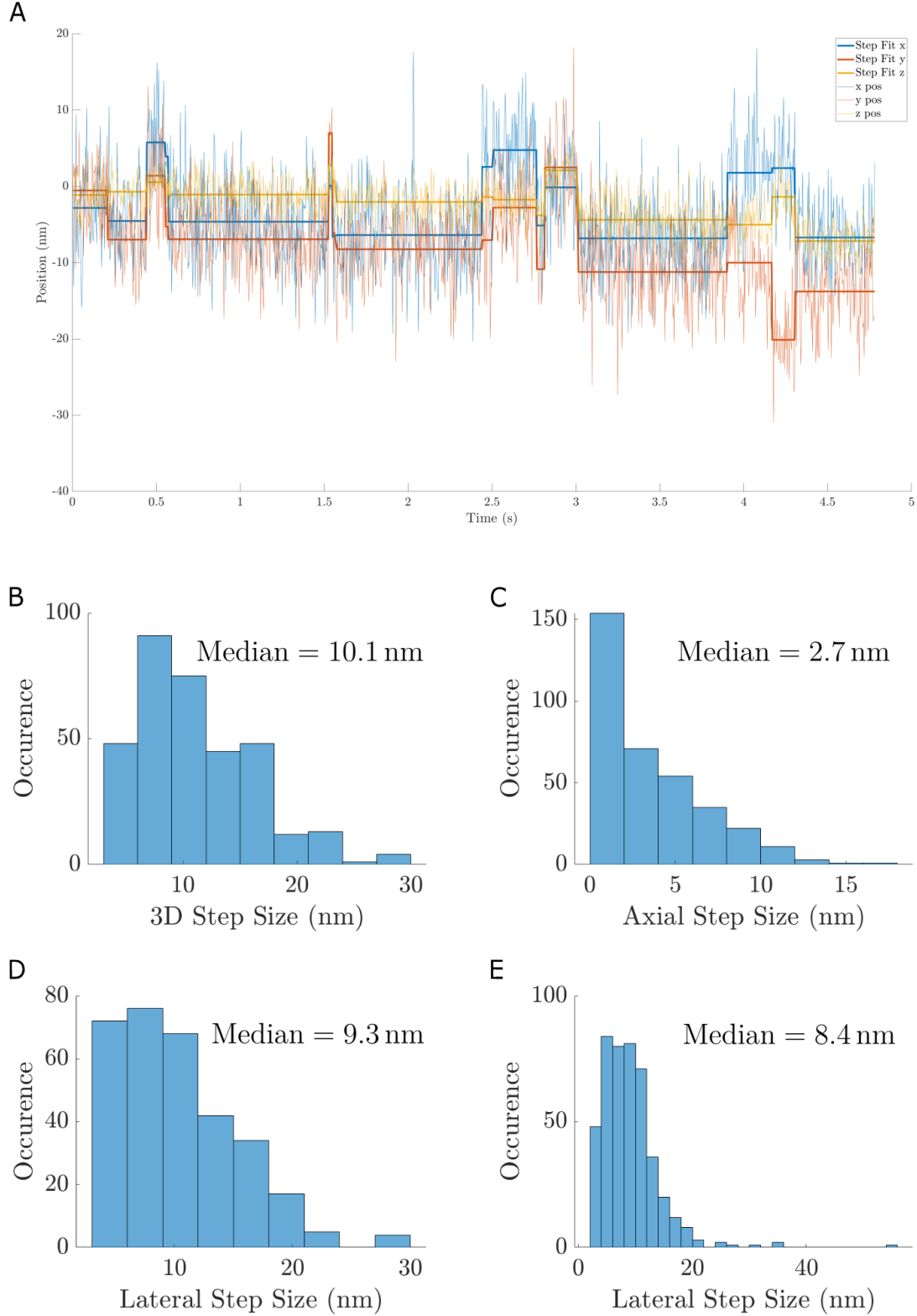


Figure 4.6: **3D MINFLUX tracking of DNA Nanorobots:** **A.** Measured Nanorobot trace with step fit for all three laboratory axes. **B.** Distribution of 3D step sizes for all measured Nanorobot traces with a median of 10.1 nm. **C.** Distribution of axial step sizes for all measured Nanorobot traces with a median of 2.7 nm. **D.** Distribution of lateral step sizes for all measured Nanorobot traces with a median of 9.3 nm. **E.** Distribution of lateral step sizes for all Nanorobot traces measured by Dr. Tobias Engelhardt⁵ on a 2D-MINFLUX setup with a median of 8.4 nm.

In total, 25 Nanorobot traces have been recorded and used for the analysis presented above. Additional example traces are compiled in Appendix D. All traces have been investigated regarding the achieved localization precision and photon efficiency. The respective values are stated in Table 4.2.

Axis	$\bar{\sigma}$ (nm)	$\bar{N}$	$\bar{\sigma}_L$ (nm)
Beam 1	2.8	22.9	13.4
Beam 2	3.1	36.5	18.7
Beam 3	3.2	28.3	17.0
Beam 4	2.6	31.7	14.6
x-axis	5.6	32.2	31.9
y-axis	5.4	32.2	30.5
z-axis	2.6	32.2	14.8

Table 4.2: **Localization precision and photon efficiency for Nanorobot measurements:** The mean localization precision and photon efficiency of the measured traces is shown. Both for the measurement in the beam coordinate system and for the measurement transformed into the laboratory coordinate system. The mean photon number for the measurement in the laboratory coordinate system is obtained as the mean from the measurements along beam axes 2-4, as only these measurements are used to compute the position in the laboratory system.

The photon efficiency achieved with this measurement is better than the efficiency achieved on single immobilized emitters with σ_L ranging from 13.4 nm to 18.7 nm in the beam coordinate system. This is because a better intensity zero could be obtained for the Nanorobot measurement, leading to a higher SBR and, therefore a better localization precision. According to Equation 2.25, the 3D localization precision in the laboratory system is 4.3 nm. The corresponding photon efficiency is 24.2 nm, which is about 40% better than was obtained in the best 3D MINFLUX implementation published to date [56].

4.4 3D Diffusion Tracking

The 3D tracking capability and the high photon efficiency of the 3D-4Pi MINFLUX were used to gain insights into 3D diffusion at the nanoscale. For this, 3D single particle tracking of small fluorescent beads and single fluorophores in solution was performed.

In general, diffusion is characterized by the diffusion coefficient D , which is related to single particle movement in the following way [74]:

$$D(t) = \frac{\text{MSD}(t)}{2mt} \quad (4.2)$$

with $\text{MSD}(t)$ being the mean squared displacement of a particle after time t and m the dimensionality of the space into which the particle is diffusing. The diffusion coefficient can take different forms depending on the kind of diffusion, such as obstructed, purely stochastic, and driven movements. In the case of thermally governed stochastic movement of particles in a liquid, the diffusion coefficient is given by the Stokes-Einstein relation

$$D = \frac{k_B T}{6\pi\eta R_0}. \quad (4.3)$$

Here, k_B is the Boltzmann constant, T and η are the temperature and the macroscopic viscosity of the fluid, and R_0 is the hydrodynamic radius of the diffusing particle. Thus, a particle's diffusion coefficient in a liquid can be tuned by altering either the particle's size, the temperature, or the liquid's viscosity, which also depends on temperature.

Plugging Equation 4.2 and 4.3 together, one can calculate the expected mean squared displacement of a particle in a given liquid for the case of free diffusion. For example, for a fluorophore with a size of about 1 nm diffusing in water, with a viscosity of 1 mPa s at room temperature, one gets a displacement of $1300 \mu\text{m}^2/\text{ms}$. This means that the particle will have traveled a net distance of several μm in the course of 1 ms, the time necessary to achieve single-digit nanometer precision in a MINFLUX measurement. Therefore, larger particles or fluids with a high viscosity need to be employed to bring the displacement of the particle into the realm where nanometer precision is possible. Orders of magnitude larger particles defy the goal of measuring diffusion on a nanoscale level, so we chose to perform measurements in high-viscosity fluids. Oversaturated sucrose-water solutions proved to be a well-suited system with viscosities ranging from several Pas up to hundreds of Pas depending on the sucrose-to-water ratio while also having a refractive index close to the immersion oil required by the microscope's objectives.

Sample Preparation⁶

Oversaturated sugar solutions containing different fluorescent markers were prepared on a chemical bench before mounting them on a coverslip. The solutions had a sucrose (S7903, Sigma-Aldrich, Merck, Darmstadt, Germany) to ultrapure H_2O ratio of w/w 83/17 to obtain a viscosity on the order of $\eta = 10\text{-}100 \text{ Pa}\cdot\text{s}$. Fluorescent markers were added to the mixture in the desired concentration. The exact mass fractions of the two components were 7.5 g of H_2O and 36.6 g of sucrose.

For the experiments with single fluorophores, we added 130 amol LD655 (LD655-NHS, Lumidyne Technologies, Brooklyn, US) diluted in 1 μL ultrapure H_2O to the sugar solution. For the experiments with fluorescent nanobeads, we used Crimson beads (FluoSpheres Carboxylate modified microspheres (Crimson), Sigma-Aldrich, Merck, Darmstadt, Ger-

⁶Sample preparation was carried out together with Dr. Michael Dasbach who was a PostDoc in our group.

many) with a diameter of 27 nm. A solution was obtained by mixing the ultrapure H₂O with 1 μ L of the Crimson beads stock solution.

The ingredients were mixed in a 50 mL flask mounted to a reflux apparatus to keep the water loss at a minimum and ensure reproducibility. The flask was heated to 120 °C while being stirred with a stirring bar at 550 rounds per minute. After 1 h, the heating was turned off, and the resulting viscous liquid was immediately poured into a conical plastic tube standing in an ice bath. This was done to prevent spontaneous crystallization, which is more likely at higher temperatures. Solutions were stored in the fridge at 4 °C for several days.

Before mounting the sugar solution, the coverslip was functionalized according to Section 3.7. Then, 50 μ L GNP 1:100 from stock in PBS was incubated on the surface for 5 min. The GNP were washed with 1 ml PBS, and the coverslip was dried under nitrogen flow. After that, a small amount of the sugar solution was added to a 15 mm coverslip, which was then put on top of the GNP-functionalized coverslip. Afterward, the sandwich was wrapped in one layer of cloth and pressed in a vise. The last step ensured a homogeneous distribution of the sugar solution and got the sample thickness as low as possible.

Theoretical background

Equation 4.2 describes the diffusion on the single particle level. Here, it will be outlined how MSD(t) is obtained from a single particle trajectory and how the measurement itself influences it. Furthermore, the two different types of diffusion observed in this study are described.

Given a time trace with observations $\vec{r}_i$ interrupted by blinking events, one can calculate the mean squared displacement (MSD) for a given time delay $n\delta_t$

$$\text{MSD}(n\delta_t) = \frac{1}{\sum_{i=1}^{N-n} a_i a_{i+n}} \sum_{i=1}^{N-n} (\vec{r}_{i+n} - \vec{r}_i)^2 a_i a_{i+n}. \quad (4.4)$$

Here, δ_t is the time between two observations and $a_i = 1$ if the emitter is on at sample i and $a_i = 0$ otherwise. N is the total number of measurements and n is the temporal distance between two measurements divided by δ_t .

For freely diffusing particles, the MSD increases linearly with time, i.e., the diffusion coefficient D becomes independent of time. Therefore, Equation 4.2 is simplified to

$$\text{MSD}_{\text{free}}(n\delta_t) = 2mDn\delta_t. \quad (4.5)$$

A measured MSD of a freely diffusing particle will deviate from this formula due to artifacts caused by the finite amount of time it takes to measure the continuously changing position

of the particle. These artifacts are taken into account in the equation below [75]:

$$\text{MSD}_{\text{free}}(n\delta_t) = 2mD\delta_t(n - 2R) + 2m\Delta^2. \quad (4.6)$$

Here, m is the number of dimensions in which the diffusion takes place, and Δ is the dynamic localization uncertainty resulting from the system's localization precision and spread of the fluorescence signal due to the emitter's movement. R is a factor that accounts for the motion blur caused by the particle's movement during the time of measurement and is defined as [76]

$$R = \frac{1}{\Delta_t} \int_0^{\Delta_t} S(t) (1 - S(t)) dt \quad (4.7)$$

where $S(t)$ is the integrated illumination up to time t within a single measurement. For homogeneous illumination one obtains $R = 1/6$.

The net effect of the two factors introduced in Equation 4.6 is a vertical shift of the MSD values measured compared to the ground truth values. This behavior is also observed in Monte Carlo simulations, as can be seen in Figure 4.8.

If the stochastic movement of an object is hindered in any way, the diffusion properties of the particle can differ from the behavior seen in free diffusion. For example, if the particle is spatially confined, the diffusion is characterized by [77]

$$\text{MSD}_{\text{conf}}(n\delta_t) = A(1 - \exp(-n\delta_t/\tau)) \quad (4.8)$$

with A being the average distance between two randomly chosen points inside the confinement region and τ the equilibration time. For a 3D sphere with diameter L , $A \approx 0.26L^2$ and for a cube with edge length L , $A \approx 0.44L^2$. The equilibration time is given as a characteristic length scale of the confinement region divided by the diffusion coefficient inside the region. Just like the parameter A , this characteristic length scale also depends on the region's shape. As an analytic expression for τ could not be found, it was estimated from fits to Monte Carlo simulations of confined diffusion (according to Section 4.4). Assuming spherical confinement regions, the inputs to the simulation could be reproduced in the fits when $\tau \approx 2A/D$. Plugging these parameters into Equation 4.8 results in

$$\text{MSD}_{\text{conf}}(n\delta_t) = 0.26L^2 \left(1 - \exp\left(-\frac{n\delta_t D}{0.52L^2}\right) \right). \quad (4.9)$$

Another type of diffusion occurs if confinement regions are partially penetrable, leading to a decoupling of diffusion on short and long timescales. This behavior is observed because the particle undergoes free diffusion on a short timescale but experiences boundaries on a larger timescale, slowing down the diffusion. This type of diffusion is called hop diffusion

and is characterized by a linear combination of free diffusion (Equation 4.5) and confined diffusion (Equation 4.9) [78, 79]

$$\begin{aligned} \text{MSD}_{\text{hop}}(n\delta_t) &= \text{MSD}_{\text{free}}(n\delta_t) + \frac{D_\mu - D_M}{D_\mu} \text{MSD}_{\text{conf}}(n\delta_t) \\ &= 2mn\delta_t \left(D_M + \frac{D_\mu - D_M}{D_\mu} \frac{0.26L_{\text{hop}}^2}{n\delta_t} \left(1 - \exp \left(-\frac{2mn\delta_t D}{0.52L_{\text{hop}}^2} \right) \right) \right) \end{aligned} \quad (4.10)$$

Here, D_μ is the short-term diffusion coefficient while D_M is the long-term diffusion coefficient. They are separated at a length scale L_{hop} . The diffusion modalities discussed above are schematically represented in Figure 4.7.

Taking into account the measurement artifacts discussed before, Equation 4.10 becomes

$$\text{MSD}_{\text{hop}}(n\delta_t) = 2m \left(D_M + \frac{D_\mu - D_M}{D_\mu} \frac{0.26L_{\text{hop}}^2}{2mn\delta_t} \left(1 - \exp \left(-\frac{2mn\delta_t D}{0.52L_{\text{hop}}^2} \right) \right) \right) \delta_t(n - 2R) + 2m\Delta^2. \quad (4.11)$$

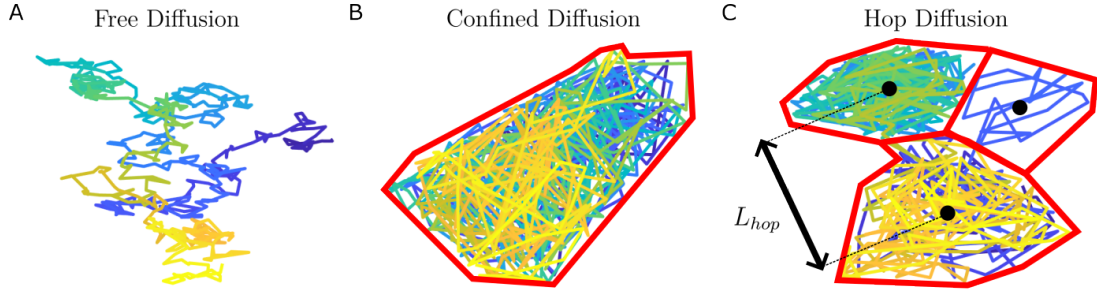
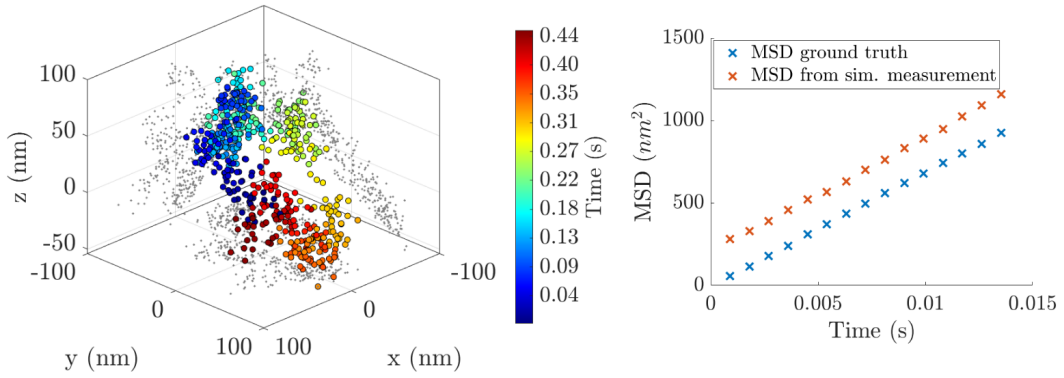


Figure 4.7: **Scheme of different diffusion modalities:** **A.** Simulated trajectory of an object experiencing free diffusion. **B.** Simulated trajectory of an object diffusing inside a confined area, illustrated by a thick red line. **C.** Simulated trajectory of an object diffusing inside of partially confined areas, whose boundaries are illustrated by thick red lines. Black dots represent the centers of the confinement areas. L_{hop} is the average distance between compartment centers.

A. Simulated free diffusion



B. Simulated hop diffusion

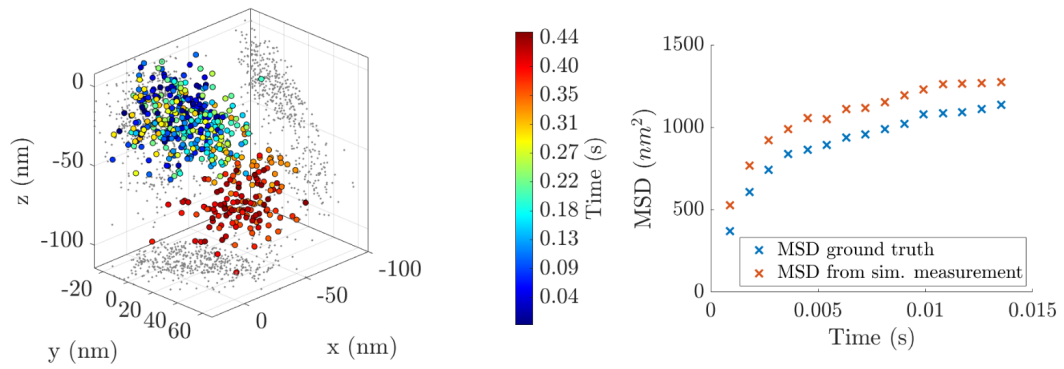


Figure 4.8: **Simulated free and hop diffusion and corresponding MSD:** **A.** Left: Simulated measured positions of a particle undergoing free diffusion with a diffusion coefficient $D = 10\,000\text{ nm}^2/\text{s}$. Right: MSD calculated from the simulated ground truth positions and from the simulated measured positions. The simulated measurement shows an offset compared to the ground truth due to the influence of the dynamic localization uncertainty (s. Equations 4.5 and 4.6). **B.** Left: Simulated measured positions of a particle undergoing hop diffusion with a diffusion coefficient $D = 100\,000\text{ nm}^2/\text{s}$, a compartment size of $L_{\text{hop}} = 30\text{ nm}$ and a hopping probability of $p_{\text{hop}} = 0.005$. Right: MSD calculated from the simulated ground truth positions and from the simulated measured positions. Again, the simulated measurement shows an offset compared to the ground truth due to the influence of the dynamic localization uncertainty (s. Equations 4.10 and 4.11).

Simulation and Analysis

An analysis routine for determining the type and magnitude of the observed diffusion was developed to quantify the results of the diffusion measurements. This analysis routine was first thoroughly tested on Monte Carlo simulations to determine the limits of its validity.

Simulation Simulations were carried out with a custom-written MATLAB code. The diffusion was simulated as a Wiener process in a 3D space divided into compartments by Voronoi tessellation. The input parameters to the simulation are the diffusion coefficient D , the radius of a Voronoi cell L_{hop} , and a probability for transitioning to a neighboring cell p_{hop} . Furthermore, the number of sampling points to be simulated and the time between

these points are given to the simulation.

As a first step, Voronoi cells are randomly distributed in space. Then, the center of a random Voronoi cell is taken as a starting point for the diffusion process. A single timestep is performed by randomly sampling from a normal distribution with mean 0 and standard deviation according to the mean distance traveled in each spatial direction given D and the time between sampling points. A step is discarded with a probability of $1-p_{\text{hop}}$ if the final position is located in a different cell than the position at the beginning of the step. Therefore, for $p_{\text{hop}} = 1$, one obtains free diffusion, and for $p_{\text{hop}} < 1$, the Wiener process becomes increasingly obstructed, leading to hop diffusion.

After that, the simulated position is linearly interpolated to increase the temporal precision such that a simulated MINFLUX measurement performed on this trace can take into account the movement of the measured object during every single exposure while also providing the same temporal precision as the simulated trace. In the next step, a simulated 3D 4Pi MINFLUX measurement is performed on the interpolated trace. The input parameters of this simulation are the spatial width and offset of the illumination function, the exposure time per illumination, and the L value of the MINFLUX pattern. First, the input trace is transformed into the beam coordinate system via a coordinate transformation previously determined in a measurement on a diffusion sample in the actual 4Pi setup. This step is taken to provide the MINFLUX measurement routine with the 'ground truth' position of the measured object in the coordinate system in which the measurement takes place. The simulated measurement is performed along three axes with three exposures each and an $L = 80$ nm. For every single exposure, several detected photons are sampled from a Poisson distribution with its expectation value according to the simulated illumination function and position of the measured object. For each cycle of nine exposures, the position of the illumination pattern is adjusted according to the same localization algorithm applied during a real measurement (described in Section 3.5). Finally, the whole trace is transformed back into the laboratory coordinate system by performing the inverse of the previous transformation. Examples of such simulated traces are given in Figure 4.8 for free diffusion ($p_{\text{hop}} = 1$) and for hop diffusion ($p_{\text{hop}} = 0.005$).

Data analysis pipeline The data analysis pipeline aims to calculate the MSD of a given trace. It is used to evaluate measured as well as simulated traces. First, each trace is cut into several sub-traces encompassing a time window of n times the sampling time δ_t . This step is taken to ensure uniform statistical sampling for each trace. A sub-trace is considered for further evaluation if at least 90% of its values fulfill the filter criteria. Those are a minimum SBR of 1 per dimension and a positive curvature according to the filter employed in the *LabView* localization routine (s. Section 3.5). Then, for each of the sub-traces, the MSD is calculated according to Equation 4.4.

Data fitting Each MSD is subjected to a fitting routine to assess whether the measured object experienced free diffusion or hop diffusion and to determine the diffusion coefficient. The fitting process and differentiation between different diffusion modes follows a similar path to the one described in [79] with the main difference that here the MSD curves are fitted instead of the diffusion coefficient curves.

Fits are performed only to the first data points of the MSD as statistical errors dominate for larger time lags. In [75], it was suggested that the diffusion coefficient is best estimated from a linear fit to only the first few data points of the MSD. At the same time, other publications state that up to a quarter of the data points can be used [80]. For the analysis performed here, 20% of data points have been used as the aim is to distinguish between free and hop diffusion, which only becomes apparent at larger time lags.

The fits are performed using the MATLAB function *fmincon*, where the sum of squared residuals (SSR) of the regression to the observed data is minimized. The fit functions are given in Equations 4.6 and 4.11 with $m = 3$ and R according to the type of data. For simulated data $\Delta = 0$ and $R = 0$, for simulated measured data $R = 1/6$ and for real measured data, $R \sim 0.15$ due to off-times during a single MINFLUX measurement which arise from switching the illumination beam and the phase of the beam. For the fit to Equation 4.11 the additional constraint of $5D_M - D_\mu < 0$ is introduced, such that free diffusion and hop diffusion are better distinguished from each other.

The values to which the fit is performed are sampled logarithmically to ensure that values for short-time separations are weighted more heavily [79] as they carry a higher statistical significance. Each fit is performed a predetermined number of times, where for each execution, new randomized starting parameters are used to prevent converging to a local minimum.

The two obtained fits are compared to each other according to their Bayesian Information Criterion (BIC)

$$\text{BIC} = n \ln(\text{SSR}/n) + k \ln(n). \quad (4.12)$$

Here, n is the number of data points and k is the number of fit parameters, i.e., $k = 2$ for free diffusion (Equation 4.6) and $k = 4$ for hop diffusion (Equation 4.11). The model with the smallest BIC is more likely and serves to categorize the data into hop or free diffusion. Once the data are evaluated, all traces categorized as free diffusion are averaged, and a fit is performed to obtain an ensemble estimate for the diffusion. The same is done for the data categorized as hop diffusion.

Test of analysis with simulated data To quantify the validity of the analysis routine, it was tested on a set of simulated traces. Here, simulated traces have been carried out with a photon emission rate and an SBR similar to that observed in the experiments. The results of these tests are summarized in Tables 4.3 and 4.4. The data were evaluated for three

		n	50	100	200
Simulation	Classification	free	94%	88%	85%
		hop	6%	12%	15%
	Results free fit	D (nm ² /s)	10000	11000	11000
		Δ_{free} (nm)	0	0	0
	Results hop fit	D _μ (nm ² /s)	22000	25000	24000
		Δ_{hop} (nm)	0	0	0
L _{hop} (nm)		17	26	37	
D _M (nm ² /s)		4400	5000	4900	
Simulated Measurement	Classification	free	84%	78%	71%
		hop	16%	22%	29%
	Results free fit	D (nm ² /s)	10000	11000	12000
		Δ_{free} (nm)	6.6	6.7	6.5
	Results hop fit	D _μ (nm ² /s)	44000	27000	29000
		Δ_{hop} (nm)	6.0	6.4	6.1
		L _{hop} (nm)	27	32	38
		D _M (nm ² /s)	6600	5400	5900

Table 4.3: **Fit results for simulation of free diffusion:** Free diffusion with a diffusion constant of $D = 10\,000\text{ nm}^2/\text{s}$ was simulated repeatedly at a sampling rate of 1.1 kHz. The resulting traces were evaluated for three different values of n (corresponding to 45 ms, 90 ms and 180 ms) according to the analysis pipeline outlined in Section 4.4. Shown here are the results of this analysis. The results specifying the mode of diffusion and quantifying the speed of the diffusion are highlighted in blue.

different values of n corresponding to a length of 45 ms, 90 ms and 180 ms respectively. Table 4.3 shows the results for the simulation of traces undergoing free diffusion with a diffusion coefficient of $D = 10\,000\text{ nm}^2/\text{s}$. The algorithm could predict free diffusion correctly for 85%-94% of the traces from the simulated ground truth for all tested values of n . The diffusion coefficient estimate is up to 20% larger than the ground truth value, and the falsely attributed result for hop diffusion gives a too-small estimate for the macro diffusion and a too-high estimate for the micro diffusion.

71%-84% of the traces from the simulated measurement could correctly be attributed to free diffusion. The diffusion coefficient was estimated to be within 20% of the ground truth value for all values of n . Furthermore, the dynamic localization uncertainty is estimated to be about 6-7 nm, which is approximately the distance the measured object covers during a single timeframe. The falsely categorized traces show a too-low macro diffusion and too-high micro diffusion.

In summary, the predicted values from the simulation and the simulated measurement are similar but differ from the ground truth. Therefore, possible deviations from expected behavior in the measurement stem from the analysis routine and not from the measurement process. The deviation of the diffusion coefficient for the simulations attributed to free diffusion is caused by traces with lower diffusion coefficients being falsely attributed to hop diffusion. The reason for the increasingly wrong classification for larger n remains

		n	50	100	200
Simulation	Classification	free	71%	38%	21%
		hop	29%	62%	79%
	Results free fit	D (nm ² /s)	44000	52000	73000
		Δ_{free} (nm)	7.1	7.0	1.9
	Results hop fit	D_{μ} (nm ² /s)	120000	120000	110000
		Δ_{hop} (nm)	4.8	6.0	6.6
		L_{hop} (nm)	42	52	63
		D_M (nm ² /s)	24000	19000	14000
Simulated Measurement	Classification	free	68%	28%	12%
		hop	32%	72%	88%
	Results free fit	D (nm ² /s)	39000	47000	60000
		Δ_{free} (nm)	8.8	8.8	7.0
	Results hop fit	D_{μ} (nm ² /s)	150000	120000	110000
		Δ_{hop} (nm)	6.0	7.5	8.0
		L_{hop} (nm)	46	52	64
		D_M (nm ² /s)	27000	18000	14000

Table 4.4: **Fit results for simulation of hop diffusion:** Confined diffusion with a diffusion constant of $D = 100\,000\text{ nm}^2/\text{s}$, a compartment size of $L_{\text{hop}} = 30\text{ nm}$ and a hopping probability of $p_{\text{hop}} = 0.001$ was simulated repeatedly at a sampling rate of 1.1 kHz. The resulting traces were evaluated for three different values of n (corresponding to 45 ms, 90 ms and 180 ms) according to the analysis pipeline outlined in Section 4.4. Shown here are the results of this analysis. The results specifying the mode of diffusion and quantifying the speed of the diffusion are highlighted in blue.

unknown.

Table 4.4 shows the results for the simulation of traces undergoing hop diffusion with a diffusion constant of $D = 100\,000\text{ nm}^2/\text{s}$, a compartment size of $L_{\text{hop}} = 30\text{ nm}$ and a hopping probability of $p_{\text{hop}} = 0.001$. Hop diffusion could correctly be attributed for 29%-79% of the simulated traces depending on the evaluated timeframe $n\delta_t$. The estimated value for D_μ is up to 20% larger than expected from the input parameters to the simulation and changes by about 10% depending on n . The estimated value for D_M changes almost by about a factor of two.

Traces from simulated measurements could be correctly attributed for over 32%-88% of the traces. The values for D_M and D_μ are similar to the results from the simulated ground truth. The value for L_{hop} is up to twice as large as the ground truth value.

Again, the analysis of simulated measurements and simulations of the ground truth show similar behavior. Therefore, deviations do not stem from the measurement process but from the analysis routine. The reason for the increase in D_M and D_μ for smaller n is caused by the increasingly wrong classification for the respective n . The reason for the wrong classification remains unknown, but the result obtained with this simulation shows that diffusion coefficients obtained from analysis of the measured data are only accurate up to a factor of two.

All in all, the analysis outlined above demonstrates the validity of the data fitting employed for traces undergoing hop diffusion in the sense that most traces are categorized correctly. Additionally, the correct value for the diffusion coefficient in the case of free diffusion is obtained up to a deviation of at most 20% (giving a quantitative result), and a separation of diffusion scales for the hop diffusion can be seen because $D_\mu/D_M \gg 5$ (giving a qualitative result).

Measurement and Results

Diffusion measurements were carried out with 27 nm diameter Crimson beads and LD655 single fluorophores at a temperature of $T = 21.5 \pm 0.5^\circ\text{C}$. LD655 demonstrates a high fluorescent rate in viscous media compared to other single molecules as it is a 'self-healing' dye. This means that it does not rely on an external buffer system to avoid long-lived dark states. This is accomplished by a Cyclooctatetraene (COT) coupled to the fluorophore, which prevents the formation of reactive oxygen species [81], making it especially useful in an environment where diffusively working buffer systems are not applicable.

The concentration of diffusing objects was adjusted so that only one object diffuses during several seconds through the excitation volume.

For finding diffusing particles, the axial position of the focus was shifted well inside the sugar solution, about 2 to 3 μm above the lower coverslip. The excitation laser was turned on, and fluorescent counts were registered on the APD until a certain threshold was

exceeded. Then a MINFLUX tracking measurement automatically started. Due to the extremely slow movement of the particles in the sugar solution, the sample was additionally shifted laterally every few scans to increase the probability of finding a fluorescent emitter. MINFLUX tracking procedures were carried out with an $L = 80$ nm along the outer three beam axes and a measurement time of $325 \mu\text{s}$ per axis.

Example traces and fit results are shown in Figures 4.9 and 4.10. More traces are compiled in Appendices E and F. For the fluorescent beads, 198 sub-traces with an $n = 200$ ($\delta_t n = 195$ ms) were evaluated, of which 89% are classified as hop diffusion. Given the discussion of the analysis routine above, this is a clear indication that hop diffusion occurred. Additionally, the traces classified as free diffusion might either be wrongly attributed or show real free diffusion. The mean fluorescence rate for all beads measurements was 59 kHz at an SBR of 3.3/3.3/3.6 along the three beam axes. The average spatial displacements for consecutive measurements along the laboratory coordinate axes were 9.2 nm/11.7 nm/6.0 nm resulting in a $\sigma_{geo} = 8.6$ nm and a photon efficiency of $\sigma_{L,geo} = 65$ nm.

For the LD655 single fluorophores, 72 sub-traces with an $n = 50$ ($\delta_t n = 49$ ms) were evaluated of which 29% are classified as hop diffusion. Therefore, it is highly likely that the single fluorophores do not exclusively exhibit hop diffusion. Furthermore, given the evaluation of simulated data, it is not justified to conclude the relative occurrence of hop diffusion to free diffusion. Nevertheless, the data presented in Figure 4.10 suggests a separation of length scales in the fluorophore's movement for the traces classified as hop diffusion. The mean fluorescence rate for all LD655 single molecule measurements was 70 kHz at an SBR of 4.8/4.4/9.0 along the three beam axes. The mean spatial displacement for consecutive measurements along the laboratory coordinate axes were 10.6 nm/11.8 nm/7.0 nm resulting in a $\sigma_{geo} = 9.6$ nm and a photon efficiency of $\sigma_{L,geo} = 79$ nm. Thus, the photon efficiency is worse for the single molecule measurement despite a higher SBR. The reason for that is the faster movement of the smaller fluorophores compared to the beads.

Below, the main results of this investigation are summarized.

- 1. Diffusion of smaller particles is faster:** The 27 nm diameter beads show a macroscopic diffusion coefficient of $770 \text{ nm}^2/\text{s}$. In contrast, the small LD655 single fluorophores show a macroscopic diffusion coefficient of $7400 \text{ nm}^2/\text{s}$ and $D = 14\,000 \text{ nm}^2/\text{s}$ from free diffusion. This is only a qualitative result as the choice of the parameter n plays a significant role in the magnitude of the evaluated diffusion coefficients.
- 2. Diffusion is separated into two scales:** Beads and potentially single fluorophores show hop diffusion, indicating a fast diffusion at short time scales up to about 5 ms and a significantly slowed down diffusion at longer time scales.
- 3. Diffusion at the nanoscale is heterogeneous:** For the nanometer-sized fluorophores, different types of diffusion could be observed, indicating that diffusion on the nanoscale depends on the nano-environment. In particular, it is shown that the environment can

significantly slow down diffusion. To our knowledge, the best explanation for this behavior is that the sugar molecules perform an oligomerization, making it harder for larger particles to penetrate through the solution. Weak self-interaction [82] and oligomerization [83] between carbohydrates have been reported before.

4. Diffusion above ~ 50 nm is in accordance with Stokes-Einstein: For the beads solution, the viscosity was measured on a rheometer (Kinexus Pro, Malvern Instruments GmbH, Herrenberg, Germany). The measurement gave $\eta = 29$ Pa s at a temperature of 22°C resulting in an expected diffusion coefficient of $D = 550 \text{ nm}^2/\text{s}$ according to Equation 4.2. This is the same order of magnitude as the experimentally determined value of $D_M = 770 \text{ nm}^2/\text{s}$.

5. Spatio-temporal precision is only limited by the speed of diffusion and fluorescence rate: Given the fact that diffusion on the nanoscale is orders of magnitude faster than expected from the Stokes-Einstein relation (Equation 4.2), nanometer precision on diffusing single fluorophores can only be obtained with a measurement modality providing a high photon efficiency. Therefore, 4Pi MINFLUX is best suited as it provides the theoretically best 3D photon efficiency, which was also demonstrated experimentally in this chapter.

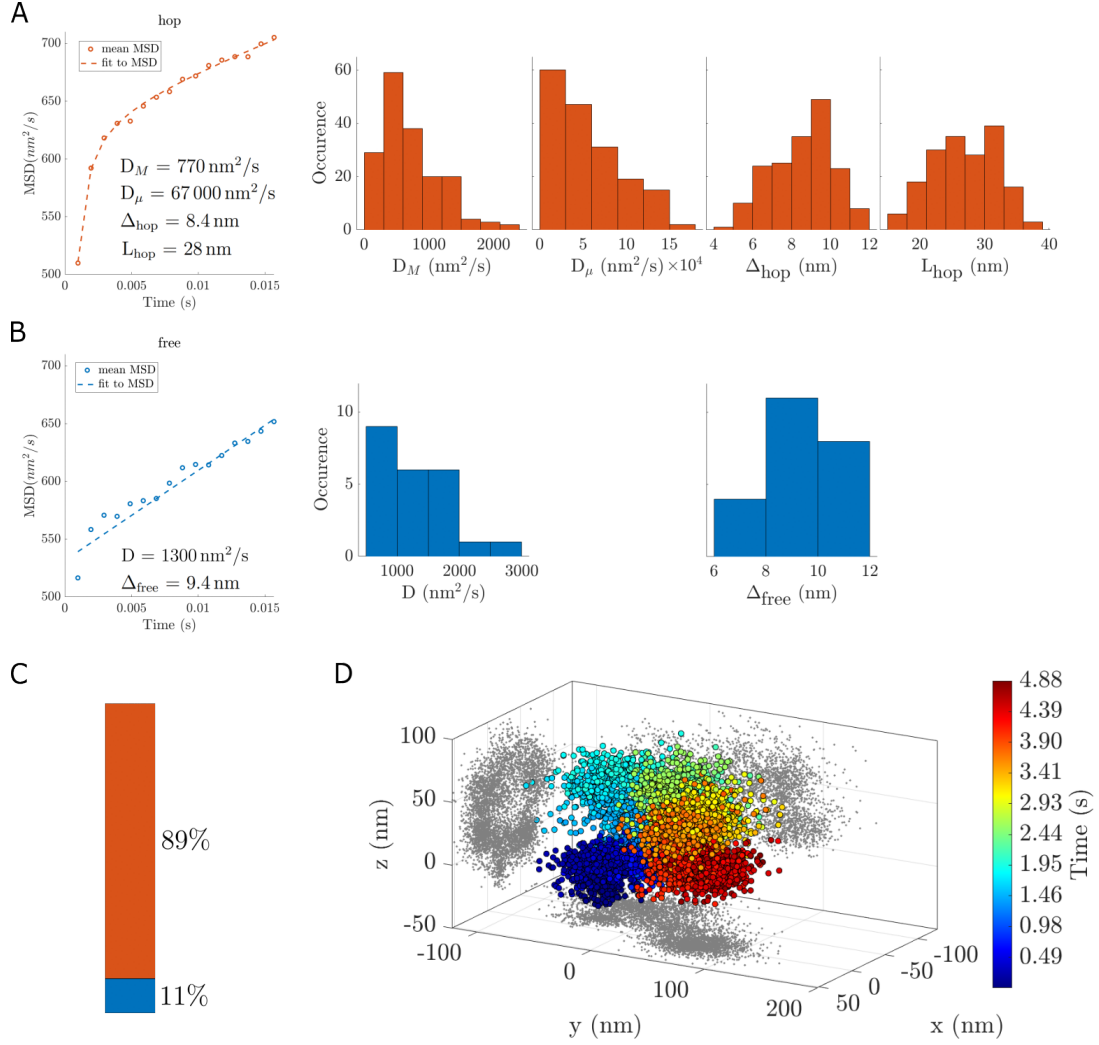


Figure 4.9: **Results from diffusion measurements on fluorescent beads:** A. Left: Average MSD of all beads traces, which are categorized as hop diffusion and corresponding fit to the data. Right: Histograms of fitted parameters for all single beads traces categorized as hop diffusion. B. Left: Average MSD of all beads traces, which are categorized as free diffusion and corresponding fit to the data. Right: Histograms of fitted parameters for all single beads traces categorized as free diffusion. C. Percentage of bead traces classified as hop/free diffusion. D. Example trajectory of a diffusing bead measured with the 4Pi MINFLUX setup.

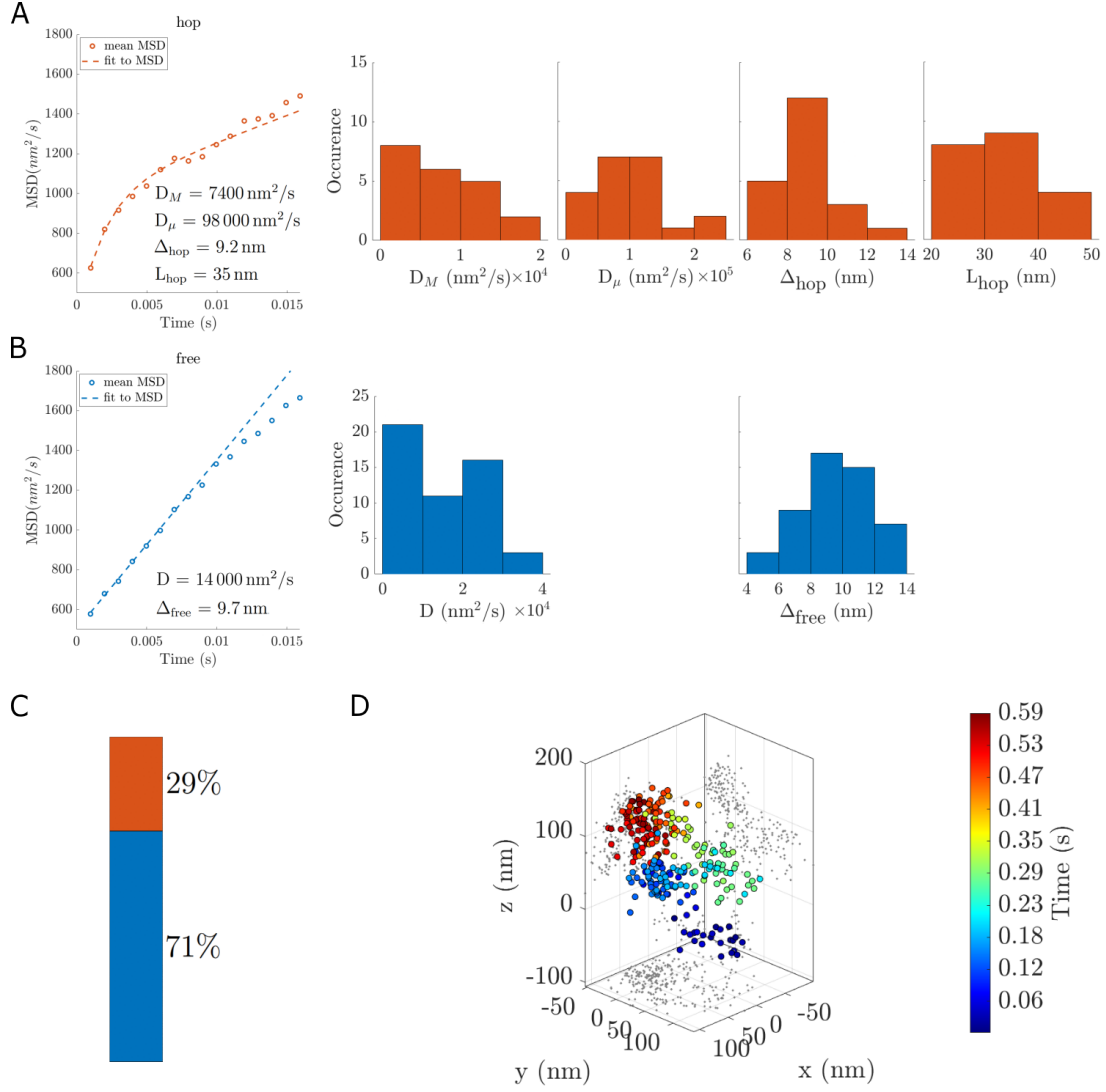


Figure 4.10: **Results from diffusion measurements on LD655 single molecules:** A. Left: Average MSD of all LD655 single molecule traces, which are categorized as hop diffusion and corresponding fit to the data. Right: Histograms of fitted parameters for all single LD655 single molecule traces categorized as hop diffusion. B. Left: Average MSD of all LD655 single molecule traces, which are categorized as free diffusion and corresponding fit to the data. Right: Histograms of fitted parameters for all single LD655 single molecule traces categorized as free diffusion. C. Percentage of LD655 single molecule traces classified as hop/free diffusion. D. Example trajectory of a diffusing LD655 single molecule measured with the 4Pi MINFLUX setup.

Chapter 5

Conclusion and Outlook

The potential of using a 4Pi setup for 3D MINFLUX tracking measurements has been investigated both theoretically and experimentally in this work. Through the use of a phase and beam scanning system specifically designed for this application, the advantage in photon efficiency predicted by calculations is translated into experimental superiority over previously published 3D MINFLUX results. The beam scanner’s flexibility enabled the illumination function to be optimized for each sample, resulting in a low intensity minimum for all measurement axes. As a result, the photon efficiency on single-molecule measurements could be increased by 40% over previous 3D MINFLUX measurements. Furthermore, a record spatio-temporal precision (STP) of 4.7 Ångström in 345 μ s for a 3D measurement was achieved at short timescales on stable, bright emitters (GNP, s. Figure 4.3). Diffusion measurements on single fluorophores and fluorescent beads, as well as tracking of DNA nanorobots, demonstrate the system’s applicability on physical and biological dynamics in the nanometer range in all three spatial dimensions with the highest temporal precision.

In MINFLUX, an emitter is localized by iteratively approaching the precisely known position of the illumination intensity’s local minimum to the emitter’s position while measuring the emitter’s response on a photodetector. In this process, the emitter’s position is probed by the local minimum in a region characterized by the length scale L , which is iteratively decreased. The curvature of the illumination intensity around the local minimum and its residual intensity in this minimum set a lower bound for L , below which the photon efficiency decreases because of insufficient contrast between observations. Therefore, an optimal MINFLUX measurement is achieved if the illumination intensity’s curvature is maximized and its residual intensity in the minimum is minimized. For tracking measurements, a well-chosen illumination function is essential, as it allows better utilization of

photons throughout the tracking process, which is performed at the optimal L after the iterative zoom-in sequence. In a 4Pi setup, the curvature of the illumination intensity is larger than in any single-lens setup, making it particularly suitable for a MINFLUX application. However, the residual intensity in the local minimum is more challenging to control than in a single-lens setup due to the setup's many components introducing residual aberrations and high requirements on the system's stability. In order to minimize the influence of residual aberrations and optimize control, the phase scanner and beam scanner presented in this thesis were developed.

The phase scanner was constructed to imprint the phase information onto the beam's polarization using electro-optic crystals before splitting the beam in the 4Pi interferometer. This minimized the number of components inside the interferometer, consequently reducing sources of potential aberrations and differential phase drifts. In addition, the phase scanner was designed to minimize the effect of temperature variations on the resulting phase difference. As a result, a phase drift of less than 2 nm is observed over a period of more than 2 hours, which is two orders of magnitude less than the drift caused by the rest of the interferometer's beam path.

The beam scanner is based on acousto-optical modulators that can shift the position of the illumination beam within a few μs . Combined with the phase scanner, this results in switching times on the μs scale between illuminations. Thus, the setup shown in this work enables the fastest 3D MINFLUX measurements to date since other 3D MINFLUX setups still rely on mechanically adjustable components such as electrically tunable lenses and deformable mirrors. In addition, unlike a rigid setup, the implementation with crystal optics provides a fast and straightforward way to adjust the individual beam pair's position to optimize the residual intensity in its local minimum. The influence of the sample and other varying experimental conditions, such as drifts due to temperature fluctuations and mechanical vibrations, make this process a daily necessity. Thus, the beam scanner substantially contributes to the performance of 3D-4Pi MINFLUX measurements.

The two scanners, together with the 4Pi illumination scheme, achieved a remarkable short-term stability that allowed a 4.7 Ångström 3D localization precision on a bright emitter at a rate of 8.7 kHz per axis, setting a record for the STP achieved in a MINFLUX measurement. Furthermore, the beam scanner enabled accurate calibration of the intensity minima along the individual measurement axes, allowing the setup to routinely achieve photon efficiencies in single-molecule measurements that outperform the best single-objective 3D MINFLUX measurements by 20%-40%.

The setup enabled the study of the 3D stepping behavior of ATTO647N-labeled DNA nanorobots in an aqueous solution, which was previously only measured with a 2D-

MINFLUX machine. Residual aberrations could be minimized by using water immersion objectives in conjunction with a flow chamber sample preparation. The stepping could be observed on tracks with a duration of up to 12 s, where steps in the expected range of 6-16 nm could be clearly resolved in 3D as evaluated with a custom-written step-finding algorithm.

The measurements show that the nanorobots move primarily in the lateral plane, where the evaluated step sizes agree with previous measurements. Moreover, changes in position along the axial direction account for a minor portion of the total step size. These axial displacements could be caused by the DNA origami not lying completely flat on the sample holder, possible inaccuracies in origami folding, and ssDNA strand flexibility. Overall, the results show that with objectives employing immersion media matched to the sample medium's refractive index, the 4Pi system can provide highly accurate 3D information in samples with refractive indices similar to water, allowing biological samples to be investigated with high STP.

The diffusion of fluorophores and fluorescent beads in highly viscous sugar solutions was studied using the 4Pi setup, which measured their 3D stochastic movement with single-digit nanometer precision. The measured traces provided ample statistics to deduce the properties of the inherent diffusion processes. A custom-written analysis script was implemented to categorize and quantify diffusion. Monte Carlo diffusion simulations in confined areas and simulations of the measurement process were used to validate the analysis.

As a result, hop diffusion, a process previously observed only in crowded environments such as the interior of cells or on the cell membrane, was observed for beads and single fluorophores in a pure sucrose- H_2O solution. Thus, the study finds evidence for compartmentalization in this solution, which hinders the movement of nanoparticles on time scales > 5 ms and corresponding length scales of > 50 nm. In contrast, movement on the nanoscale is significantly faster. The observed behavior can be explained by weak self-interaction and oligomerization between the sugar molecules, which has been reported before for other carbohydrates. Also, it was confirmed that the diffusion velocity on large scales is particle size dependent and in agreement with the value predicted by the Einstein-Stokes relation.

The calibration and benchmark measurements and the biological and physical studies conducted in this thesis demonstrate the exceptional photon efficiency and associated high STP of 3D-4Pi MINFLUX. Thus, highlighting its significance for investigating various questions in life sciences and beyond.

Nevertheless, the 3D-4Pi MINFLUX setup presented in this work still shows room for

advancement. Although an improvement in photon efficiency down to $\sigma_L = 9.8$ nm for 3D MINFLUX measurements was demonstrated, it does not reach the efficiency reported for state-of-the-art 1D MINFLUX ($\sigma_L = 4.5$ nm in [64]) and 2D MINFLUX measurements ($\sigma_L = 5.5$ nm in [63]). The main reason for this is the difficulty in ensuring a low intensity minimum for all three measurement axes at the same time, which is why the minimum L in this work was only 20 nm and 40 nm on a regular basis. Therefore, it is of particular importance to implement an optimized zoom-in process, where the subsequent L is chosen dynamically based on the information already collected. This allows all three axes to be tuned to their individually optimal L during a measurement.

In addition, it is expected that the quality, as well as the stability of the intensity minimum, will be improved by better passive as well as active setup stability, which can be achieved by external monitoring and adjustment of the objective positions with an additional infrared interferometer, active temperature stabilization of the entire setup, and reduction of setup size down to a monolithic assembly. Additionally, drift of the sample can be compensated by active sample stabilization via fiducial markers or a grating imprinted onto the coverslip as has been demonstrated in [63].

In the end, however, the influence of the sample remains, which is especially critical for 4Pi, since the phase of at least one of the two beams is inevitably modified by it, and reflexes occur at the sample/glass interface, which is close to the focal spot. In MINFLUX, where measurements with an accuracy of up to 1/1000th of the excitation wavelength are performed, these inaccuracies may prove to be the limiting factor in achieving a low intensity minimum.

On the other hand, this work demonstrated that the influencing factors are small enough to allow for a better photon efficiency in a 3D-4Pi MINFLUX setup than with a state-of-the-art single-lens 3D MINFLUX setup. Also, the photon efficiency in 3D-4Pi MINFLUX can be enhanced using smaller illumination beams and placing them further to the outer edge of the objective's back focal plane, as has been shown in Chapter 2.2. Therefore, it is suggested to test the previously described improvements in a 3D-4Pi MINFLUX setup to push the limits of the method further.

Eventually, the 4Pi setup is designed for multi-color measurements, as the electro-optical and acousto-optical control allows to switch between different wavelengths within μ s. The optical setup and corresponding software can also perform imaging measurements with the help of an activation laser introduced through the detection beam path.

Appendix A

Transformation between laboratory system and 3D-4Pi beam system

For performing 3D localizations in a 4Pi system, the position is obtained along the axes of tilted beams. As this beam system does not need to be cartesian, i.e. the angle between each pair of axes is not necessarily 90° , a coordinate transformation into the cartesian laboratory system needs to be performed to obtain the true 3D position of a measured object. For the following derivation it is assumed that the three beam pairs are equidistantly spaced on a circle with radius r around the optical axis.

The following transformation matrices will be employed for the coordinate transformation:

$$R_z(\alpha) = \begin{bmatrix} \cos(\alpha) & -\sin(\alpha) & 0 \\ \sin(\alpha) & \cos(\alpha) & 0 \\ 0 & 0 & 1 \end{bmatrix} \quad (\text{A.1})$$

$$R_x(\alpha) = \begin{bmatrix} 1 & 0 & 0 \\ 0 & \cos(\alpha) & -\sin(\alpha) \\ 0 & \sin(\alpha) & \cos(\alpha) \end{bmatrix} \quad (\text{A.2})$$

$$R_{\vec{n}}(\alpha, n_1, n_2, n_3) = \begin{bmatrix} n_1^2 \cdot (1 - \cos(\alpha)) + \cos(\alpha) & n_1 \cdot n_2 \cdot (1 - \cos(\alpha)) - n_3 \cdot \sin(\alpha) & n_1 n_3 \cdot (1 - \cos(\alpha)) + n_2 \cdot \sin(\alpha) \\ n_1 \cdot n_2 \cdot (1 - \cos(\alpha)) + n_3 \cdot \sin(\alpha) & n_2^2 \cdot (1 - \cos(\alpha)) + \cos(\alpha) & n_2 \cdot n_3 \cdot (1 - \cos(\alpha)) - n_1 \cdot \sin(\alpha) \\ n_1 \cdot n_3 \cdot (1 - \cos(\alpha)) - n_2 \cdot \sin(\alpha) & n_2 \cdot n_3 \cdot (1 - \cos(\alpha)) + n_1 \cdot \sin(\alpha) & n_3^2 \cdot (1 - \cos(\alpha)) + \cos(\alpha) \end{bmatrix} \quad (\text{A.3})$$

These matrices describe rotations of the coordinate system by an angle of α around the z-axis, x-axis and $\vec{n}$ -axis respectively. Here, $\vec{n}$ needs to be a unit vector going through the origin of the coordinate system. Finally, a shearing transformation is needed as the three beams do not necessarily span a cartesian coordinate system, i.e. the angles between each pair of axes differs from $\pi/2$. The matrix

$$S(\beta) = \begin{bmatrix} 1 & \tan(\beta) & \tan(\beta) \\ \tan(\beta) & 1 & \tan(\beta) \\ \tan(\beta) & \tan(\beta) & 1 \end{bmatrix} \quad (\text{A.4})$$

corresponds to a shear of the coordinate system, such that the new coordinate axes have an angle of

$$\gamma = \arccos \left(\frac{\cos(2\beta) - 2\sin(2\beta) - 1}{\cos(2\beta) - 3} \right) \quad (\text{A.5})$$

to each other.

A change from the initial cartesian coordinate system with the z-axis aligned with the optical axis to the 'beam system' is given by the transformation

$$T(\alpha, \beta) = R_x(\arctan(\sqrt{2})) \cdot R_z(\pi/4) \cdot R_{\vec{n}} \left(\alpha, \frac{1}{\sqrt{3}}, \frac{1}{\sqrt{3}}, \frac{1}{\sqrt{3}} \right) \cdot S(\beta) \quad (\text{A.6})$$

where α is a parameter which describes the rotation of the new coordinate system around the optical axis.

Let the new basis vectors be (λ, μ, ν) . Then, they relate to the old basis (x, y, z) via

$$\begin{aligned}
x &= - \frac{\cos(\beta) \cdot [\sqrt{3} \cdot \sin(\alpha) - 3 \cdot \cos(\alpha)]}{3 \cdot \sqrt{2} \cdot [\cos(\beta) - \sin(\beta)]} \cdot \lambda \\
&\quad - \frac{\cos(\beta) \cdot [\sqrt{3} \cdot \sin(\alpha) + 3 \cdot \cos(\alpha)]}{3 \cdot \sqrt{2} \cdot [\cos(\beta) - \sin(\beta)]} \cdot \mu \\
&\quad + \frac{\sqrt{2} \cdot \cos(\beta) \cdot \sin(\alpha)}{\sqrt{3} \cdot [\cos(\beta) - \sin(\beta)]} \cdot \nu \\
y &= \frac{\cos(\beta) \cdot [\sqrt{3} \cdot \cos(\alpha) + 3 \cdot \sin(\alpha)]}{3 \cdot \sqrt{2} \cdot [\cos(\beta) - \sin(\beta)]} \cdot \lambda \\
&\quad + \frac{\cos(\beta) \cdot [\sqrt{3} \cdot \cos(\alpha) - 3 \cdot \sin(\alpha)]}{3 \cdot \sqrt{2} \cdot [\cos(\beta) - \sin(\beta)]} \cdot \mu \\
&\quad - \frac{\sqrt{2} \cdot \cos(\beta) \cdot \cos(\alpha)}{\sqrt{3} \cdot [\cos(\beta) - \sin(\beta)]} \cdot \nu \\
z &= + \frac{\cos(\beta)}{\sqrt{3} \cdot [\cos(\beta) + 2 \sin(\beta)]} \cdot \lambda \\
&\quad + \frac{\cos(\beta)}{\sqrt{3} \cdot [\cos(\beta) + 2 \sin(\beta)]} \cdot \mu \\
&\quad + \frac{\cos(\beta)}{\sqrt{3} \cdot [\cos(\beta) + 2 \sin(\beta)]} \cdot \nu
\end{aligned} \tag{A.7}$$

Omitting the arbitrary rotation angle, the transformation from the beam system to the laboratory system only depends on the shearing angle β given in Equation 2.22.

$$\vec{x} = \begin{bmatrix} \frac{\cos(\beta)\sqrt{1+2\tan(\beta)}}{\sqrt{2}(\cos(\beta)-\sin(\beta))} \\ \frac{\cos(\beta)\sqrt{1+2\tan(\beta)}}{\sqrt{2}(\cos(\beta)-\sin(\beta))} \\ 0 \end{bmatrix} \tag{A.8}$$

$$\vec{y} = \begin{bmatrix} \frac{\cos(\beta)\sqrt{1+2\tan(\beta)}}{\sqrt{6}(\cos(\beta)-\sin(\beta))} \\ \frac{\cos(\beta)\sqrt{1+2\tan(\beta)}}{\sqrt{6}(\cos(\beta)-\sin(\beta))} \\ -\frac{\sqrt{2}\cos(\beta)\sqrt{1+2\tan(\beta)}}{\sqrt{3}(\cos(\beta)-\sin(\beta))} \end{bmatrix} \tag{A.9}$$

$$\vec{z} = \begin{bmatrix} \frac{\cos(\beta)\sqrt{1+2\tan(\beta)}}{\sqrt{3}(\cos(\beta)+2\sin(\beta))} \\ \frac{\cos(\beta)\sqrt{1+2\tan(\beta)}}{\sqrt{3}(\cos(\beta)+2\sin(\beta))} \\ \frac{\cos(\beta)\sqrt{1+2\tan(\beta)}}{\sqrt{3}(\cos(\beta)+2\sin(\beta))} \end{bmatrix} \quad (\text{A.10})$$

From these equations the uncertainty of the position estimation in the beam system can be calculated given the uncertainty from the measurements along the beam axes. This result is presented in Equations 2.23 and 2.24.

Appendix B

Specifications of RTP crystals

We use RTP crystals for our phase modulation as they exhibit a birefringent linear electrooptic coefficient and can be produced at a high quality. The crystal that we use are x-cut, meaning that the laser propagates along the x-axis. Therefore, the two polarizations of the laser oscillate along the y and z crystal axis. Furthermore, a voltage is applied along the z-axis. The relevant electro-optical parameters of RTP are given in the Table below. The values in the Table have been compiled from [84] and [85].

Property		Value
linear electrooptic coefficient (pm/V)	r_{13}	10.9
	r_{23}	15.0
	r_{33}	33.0
refractive index	n_x/n_o	1.793 (610nm)
	n_y/n_e	1.805 (610nm)
	n_z	1.895 (610nm)
	dn_x/dT	9.7 (633nm)
thermooptic coefficient ($10^{-6}/K$)	dn_y/dT	12.8 (633nm)
	dn_z/dT	23.3 (633nm)
thermal expansion coefficient ($10^{-6}/K$)	$\alpha_{T,x}$	9.44
	$\alpha_{T,y}$	12.5
	$\alpha_{T,z}$	-4.16
dielectric constant	ϵ_r	13

Table B.1: **Properties of RTP crystals:** All electro-optic and thermal values relevant for the phase scanner presented in this thesis. The refractive indices and linear electrooptic coefficients are used to calculate the half-wave voltage. Thermooptic coefficient and thermal expansion coefficient are used for calculating the temperature stability.

Appendix C

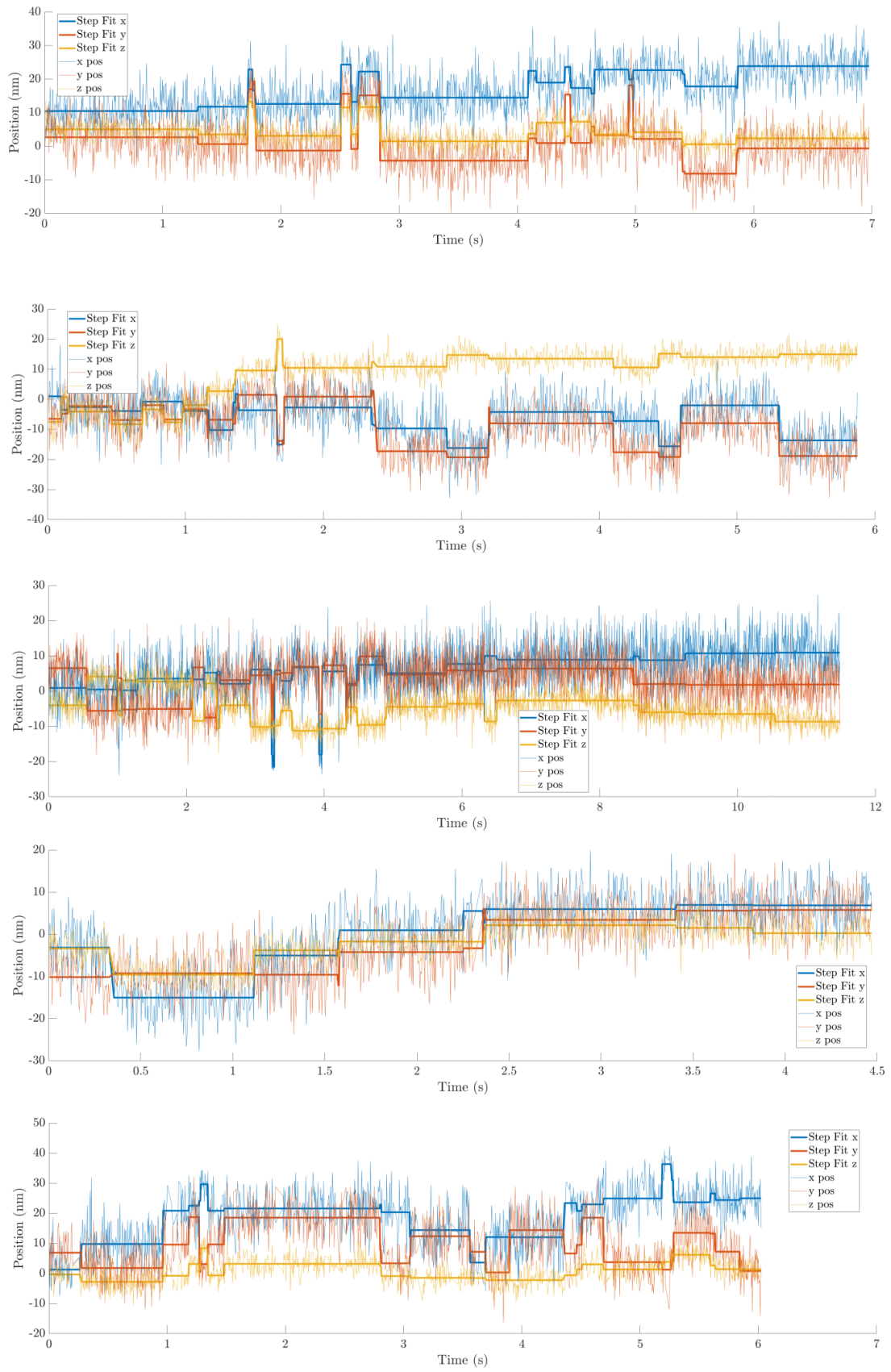
Specifications of 2D-AOD

The AODs used for the beam scanner in the 3D-4Pi MINFLUX setup are made up of TeO_2 , which has a velocity of sound of $v_s = 650\text{m/s}$. The individual crystals are cut such that a laser beam entering perpendicular to the input face will exit the crystal on the same axis if the acoustic frequency is set to the center of the bandwidth. The bandwidth for light at 450 nm is 85-110MHz and the bandwidth for light at 650 nm is 54-71MHz. The active aperture of all four crystals is 7.5 mm. The crystals are designed such that the visible spectrum from 450 nm-650 nm can be deflected within an overlapping solid angle of 18mrad^2 .

Appendix D

Additional Nanorobot traces

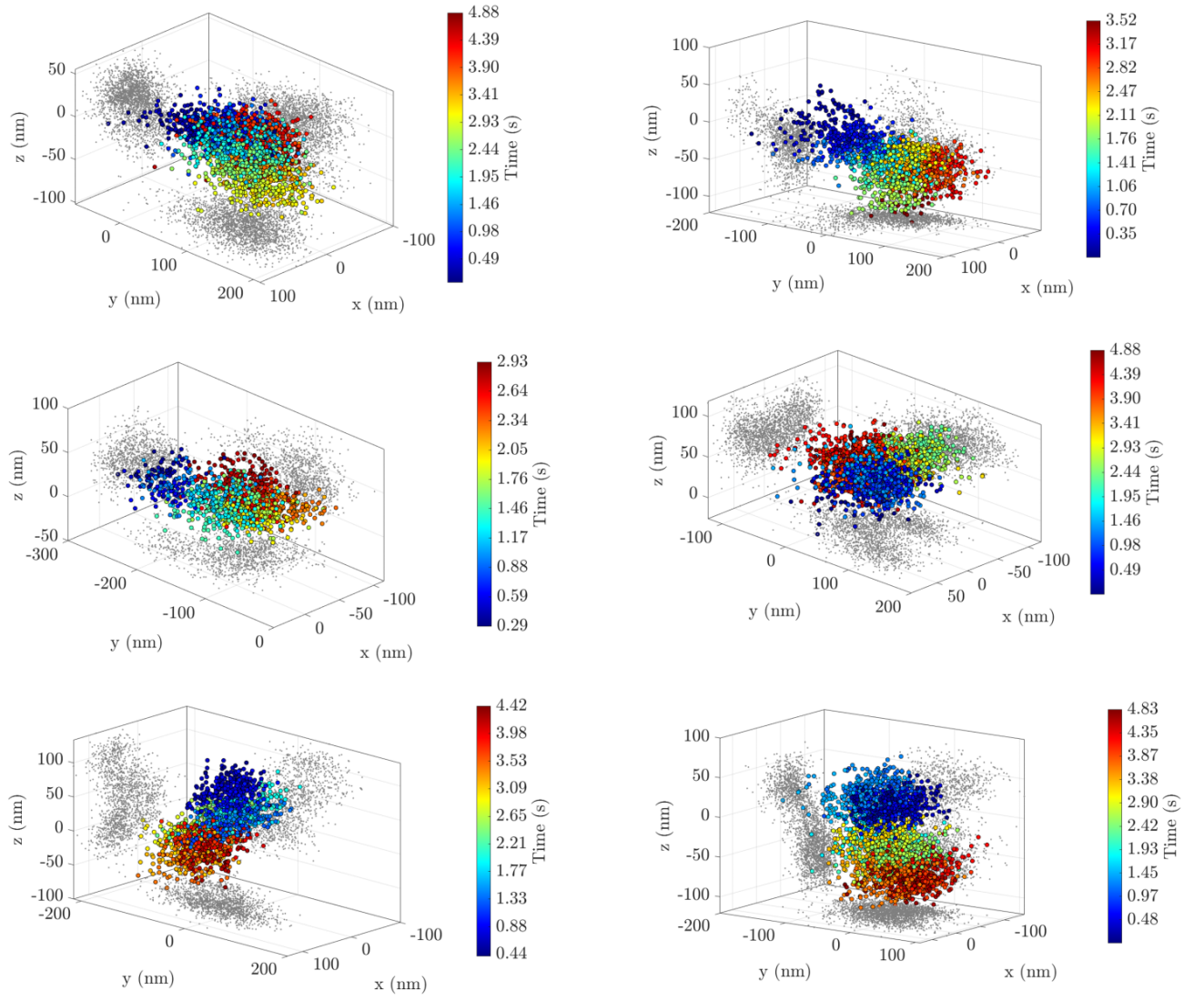
Additional 3D Nanorobot traces with step fit for all three laboratory axes. The measurements were taken in the 4Pi MINFLUX setup with an $L = 40$ nm along four beam axes with a measurement time of $925\ \mu\text{s}$ per axis. Traces shown here were used for generating the step size distributions shown in Figure 4.6.

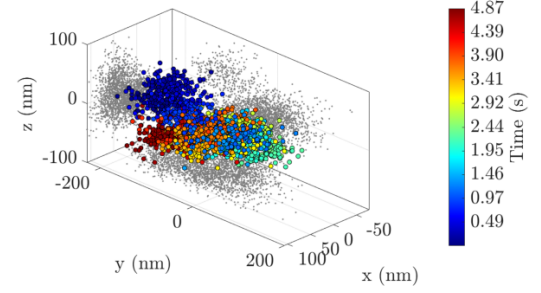
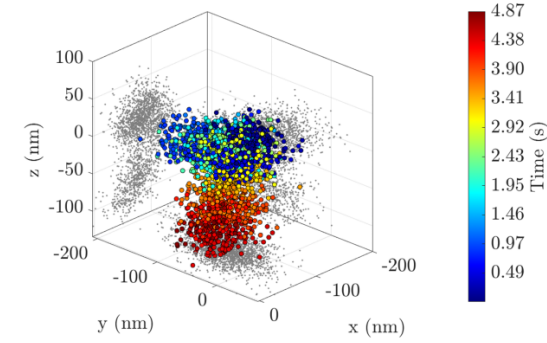
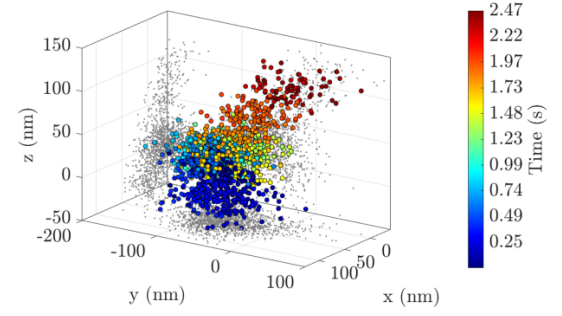
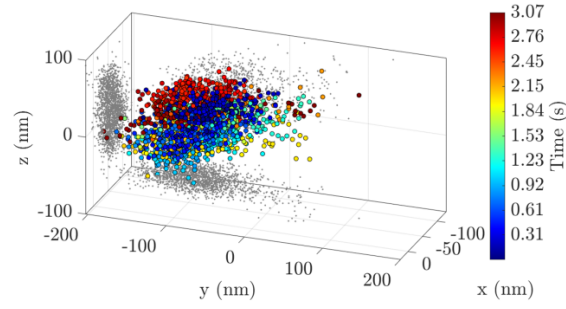
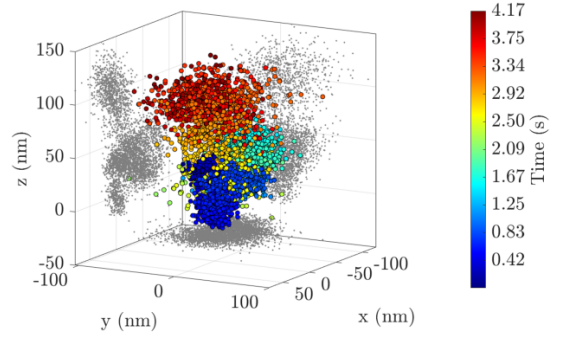
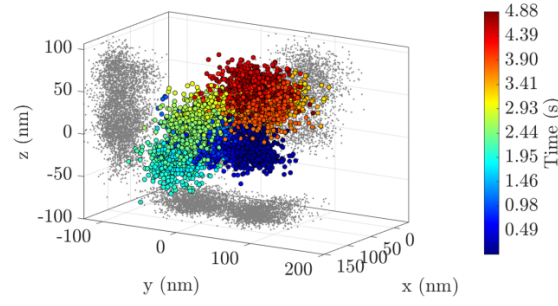
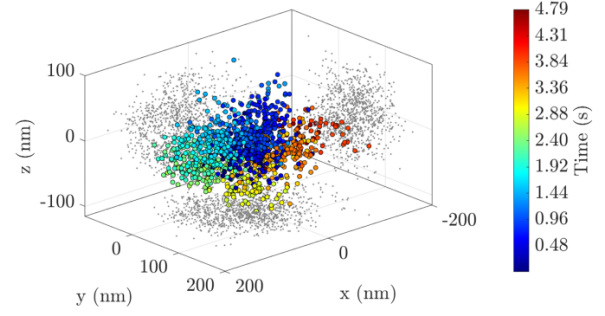
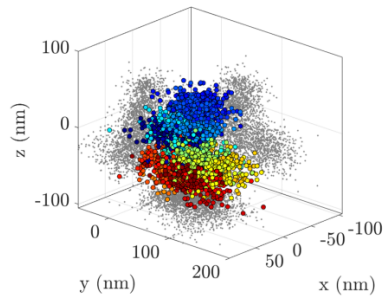
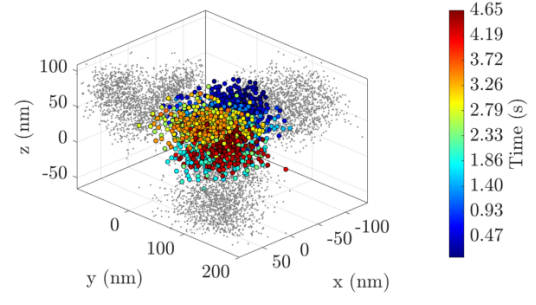
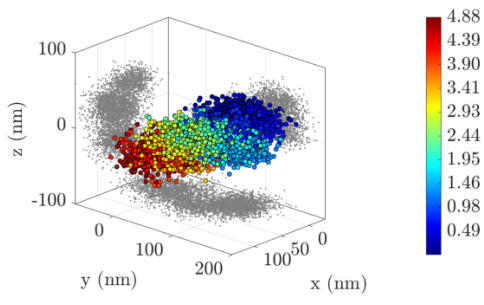


Appendix E

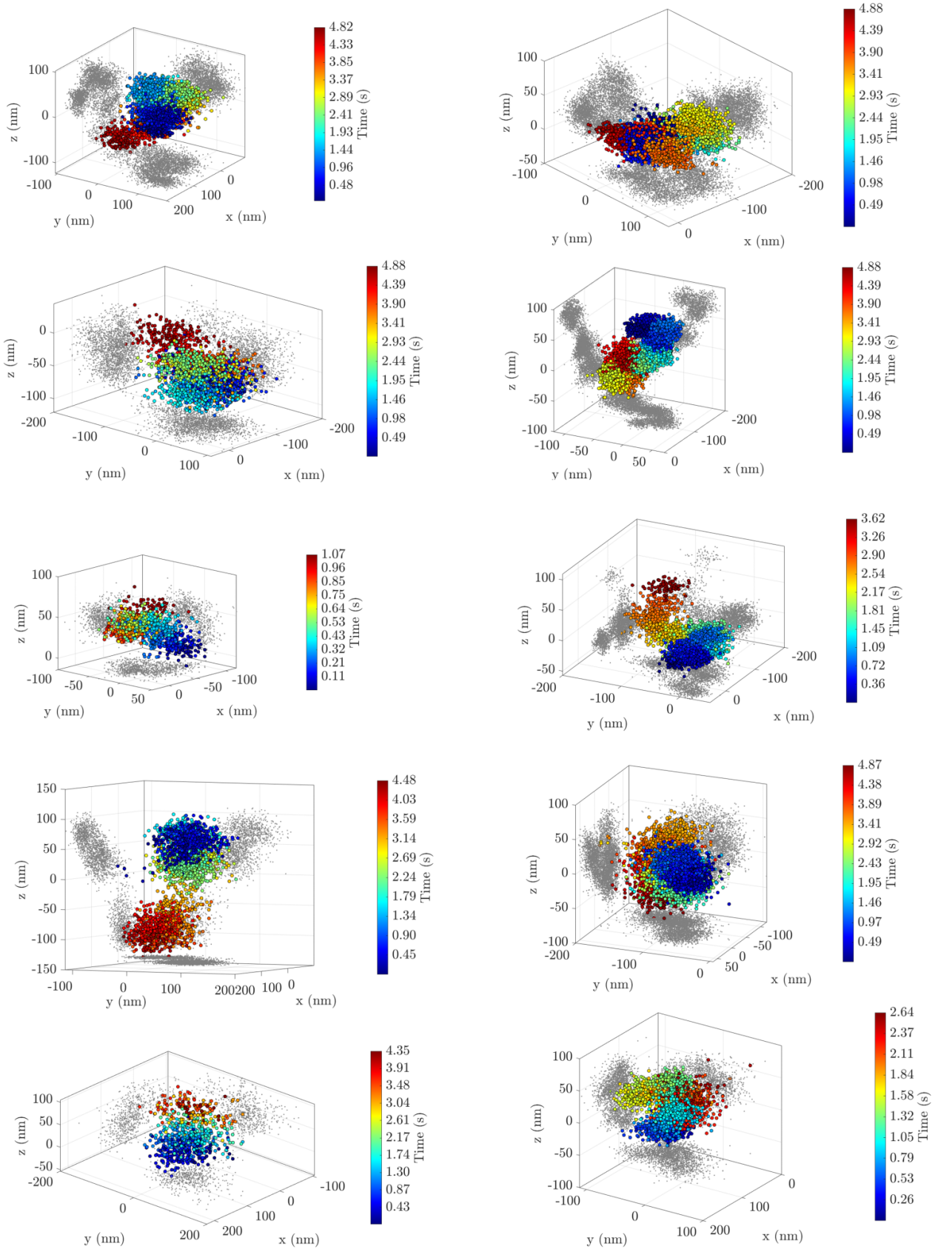
Additional traces of diffusing Crimson beads

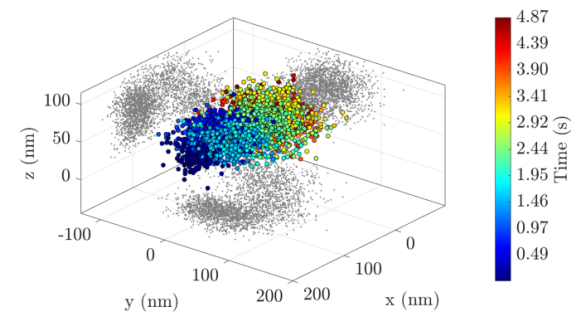
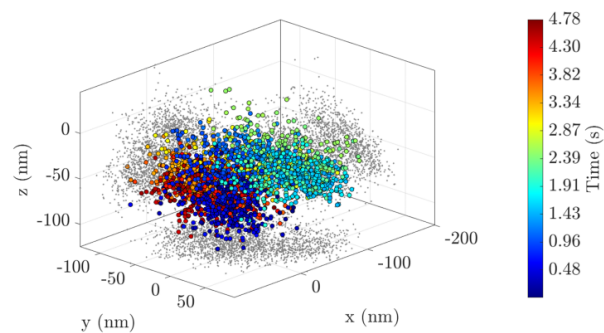
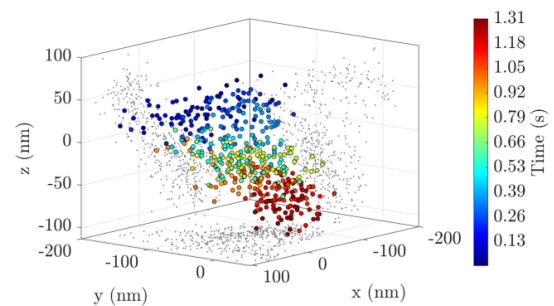
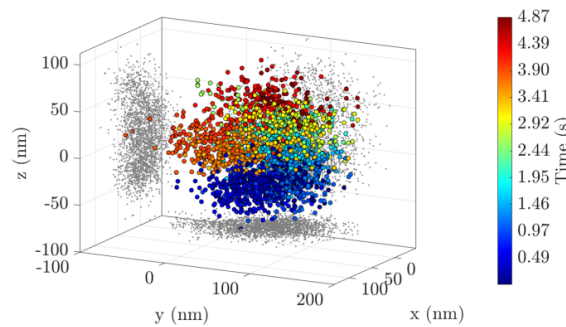
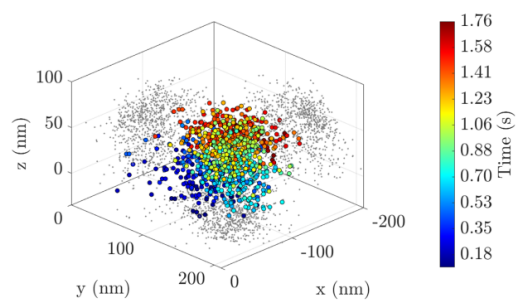
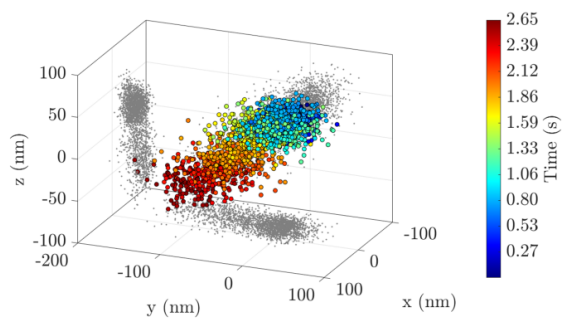
Additional 3D trajectories of diffusing Crimson beads measured with the 4Pi MINFLUX setup with an $L = 80\text{nm}$ along three beam axes with a measurement time of $325\,\mu\text{s}$ per axis.





APPENDIX E. ADDITIONAL TRACES OF DIFFUSING CRIMSON BEADS

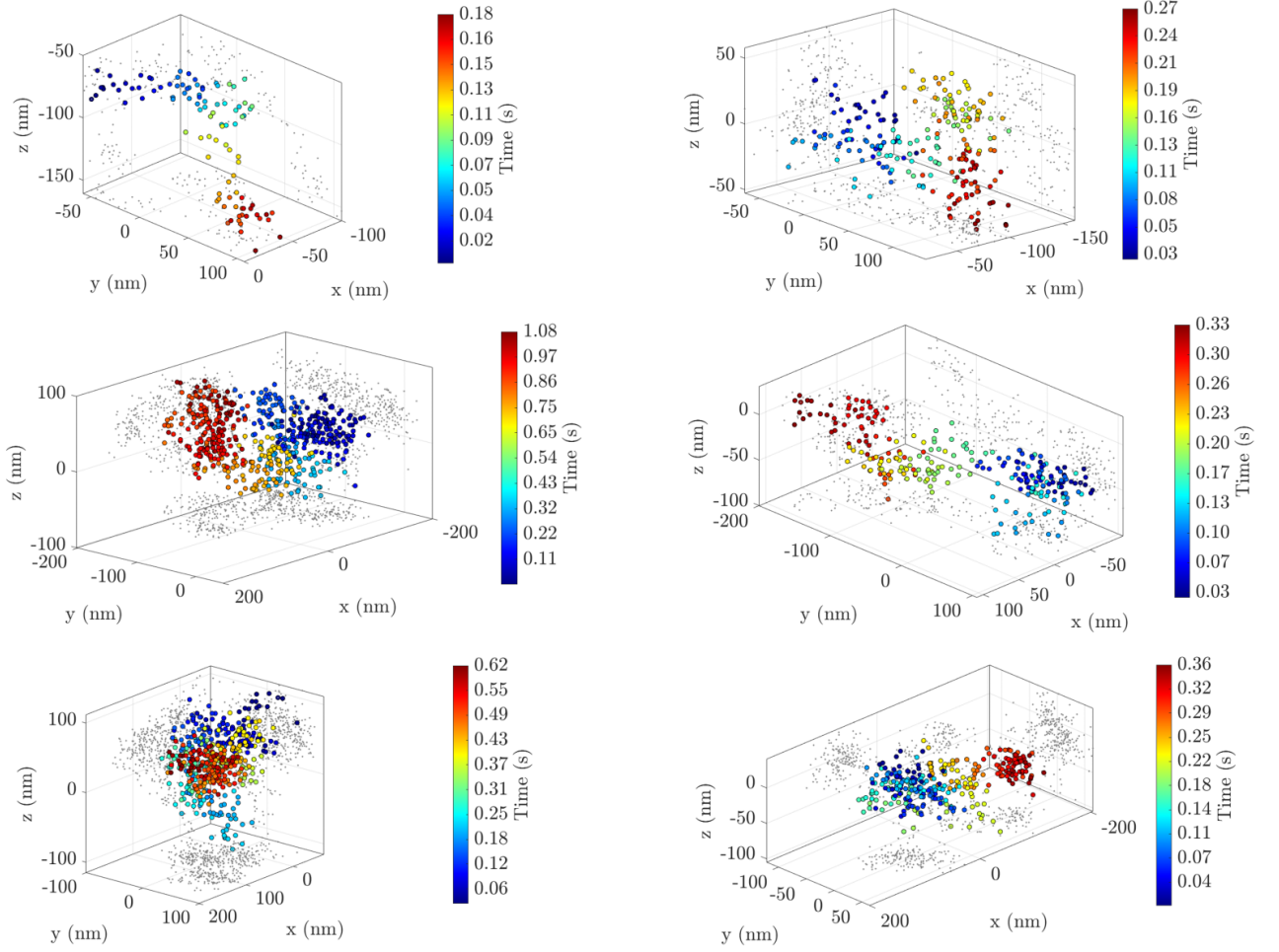


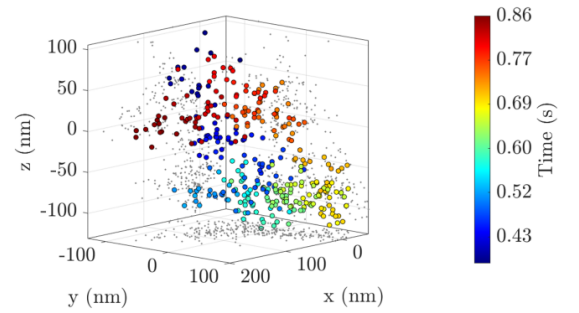
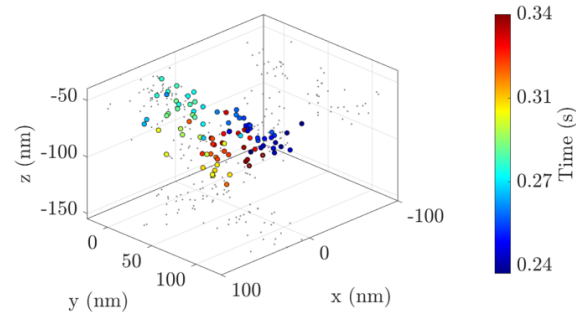
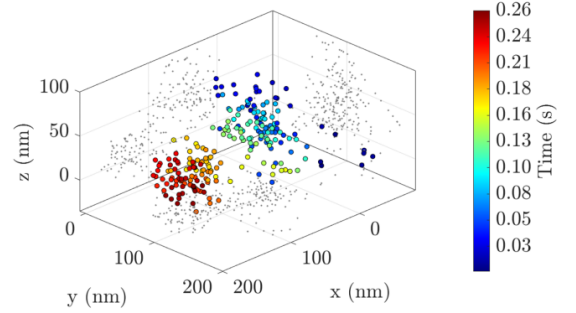
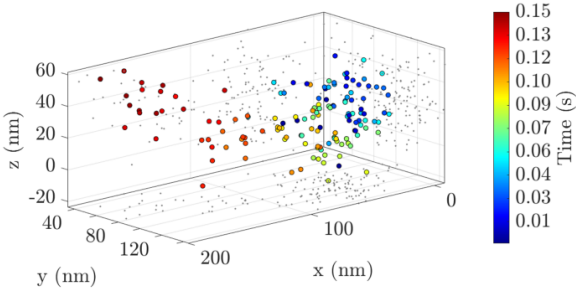
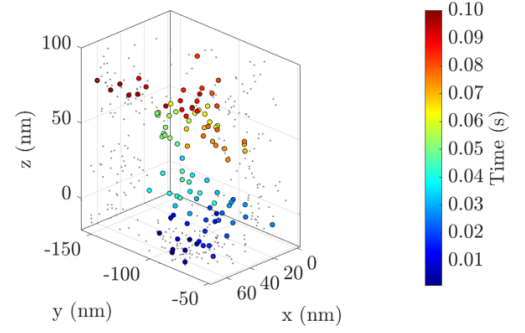
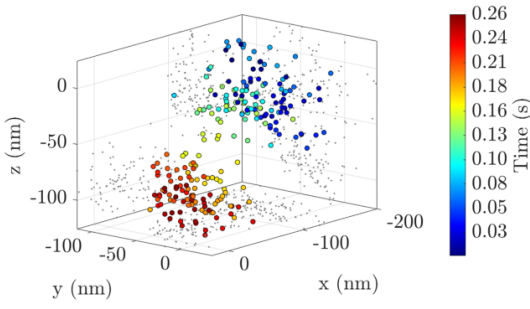
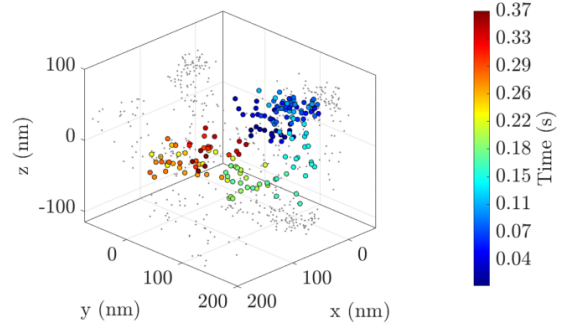
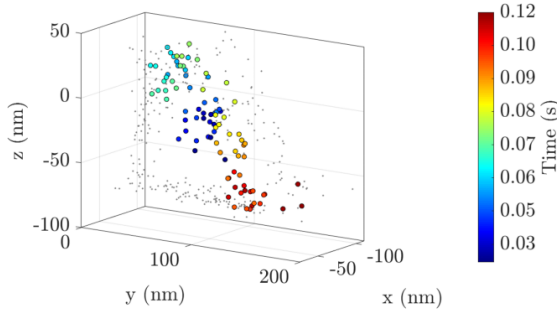
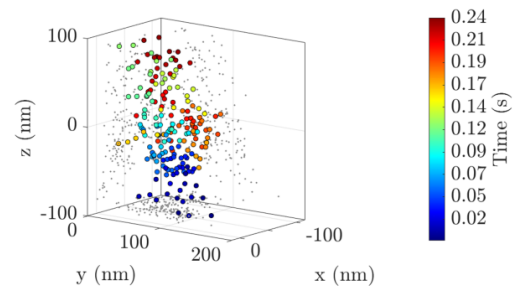
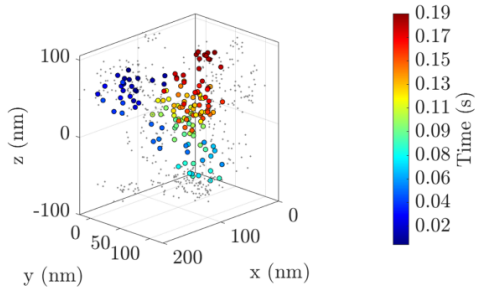


Appendix F

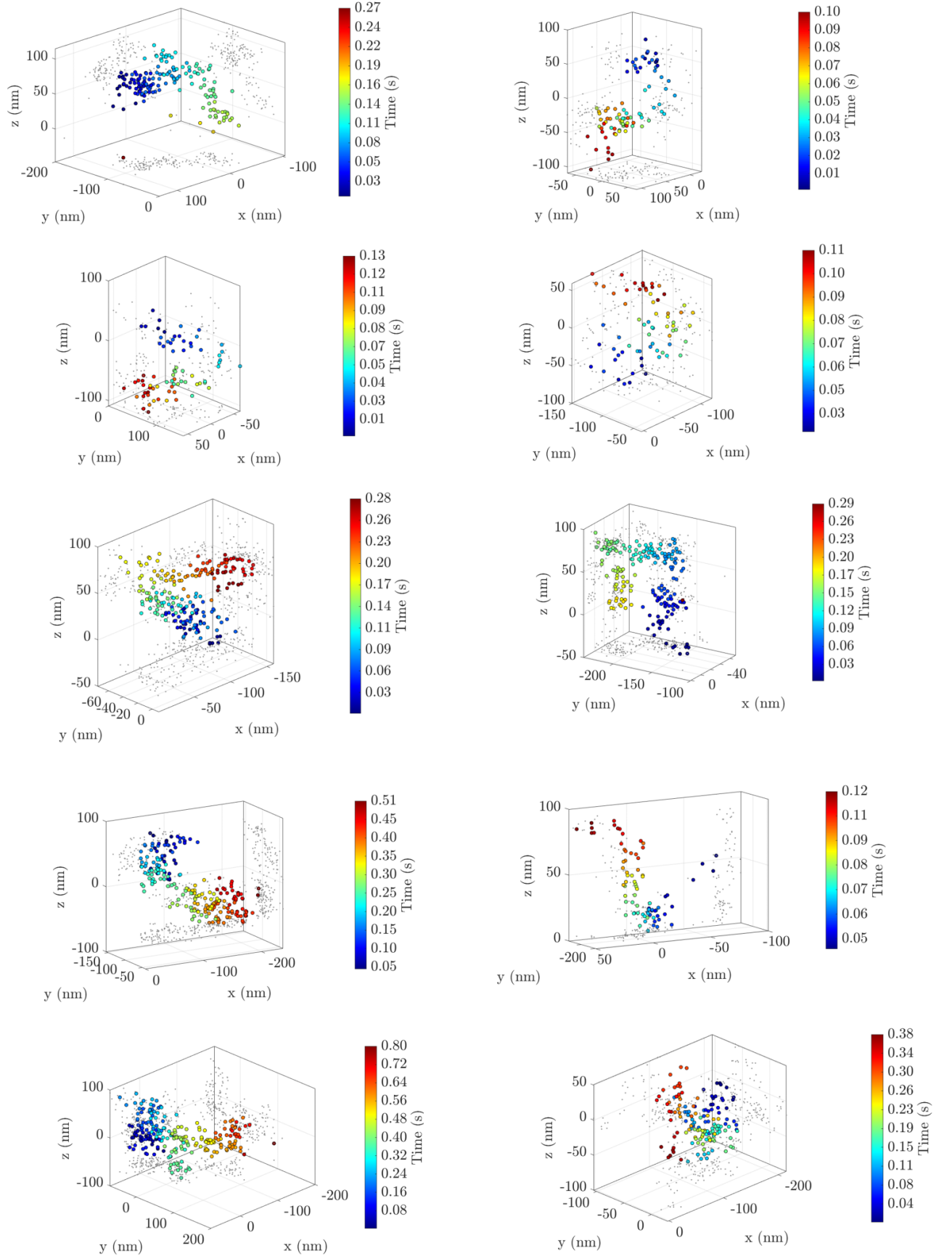
Additional traces of diffusing LD655 single fluorophores

Additional 3D trajectories of diffusing LD655 single fluorophores measured with the 4Pi MINFLUX setup with an $L = 80\text{nm}$ along three beam axes with a measurement time of $325\,\mu\text{s}$ per axis.





APPENDIX F. ADDITIONAL TRACES OF DIFFUSING LD655 SINGLE FLUOROPHORES



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