

Applications of Interferometric Scattering (iSCAT) Microscopy to Single Particle Tracking in Model and Cell Membranes



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Abstract

We have employed Interferometric Scattering (iSCAT) microscopy-based Single Particle Tracking (SPT) to study lipid dynamics of on model membranes and plasma membranes. This novel imaging technique offers high localization precision ($\sim 10^1\text{nm}$) and temporal resolution ($< msec$), together with tunable Signal-to-Noise Ratio.

In the Materials and Methods, we describe our custom iSCAT setup. Also, an advanced analysis pipeline for SPT data is introduced, to estimate camera blurring and localization error effects in SPT data, and to statistically evaluate the most likely model of particle diffusion dynamics.

In Chapter 3, we show the results of SPT experiments on gold nanoparticle-tagged lipids on model membranes. In slow sampling rate experiments (100Hz), the effects of localization error are not very pronounced, and vice versa in high sampling rate (2kHz) experiments. These experiments, corroborated by Fluorescence Correlation Spectroscopy (FCS) measurements, show that gold-tagged lipids are much slower compared to fluorescent-dye tagged ones. This effect was attributed to the crosslinking potential and large size and of the gold tag.

In Chapter 4, the effects of crosslinking are explored. Streptavidin and a monovalent version thereof (“Flavidin”) are used to tag biotinylated lipids, and their dynamics analyzed through iSCAT-SPT and FCS. Both techniques show how tetravalent Streptavidin-tagged particles are ~ 2 times slower than Flavidin-tagged ones. Detection of 2kDa polymer-conjugated lipids was also achieved with iSCAT.

Chapter 5 is focused on the results of gold-tagged saturated lipids tracking on epithelial cell membranes. Their hopping diffusion behaviour was analyzed, and the diffusion in the confinement areas was found to be much slower than what previously reported.

In conclusion, iSCAT-SPT is a very valuable technique in the study of membrane biophysics, which has helped us uncover some details on the effect of tag crosslinking, and promising details on the diffusion of lipids on cell membrane. Future developments in technology and analysis methodology will provide more insights in single molecule dynamics on membranes.

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Chapter 1

Introduction

1.1 Premise

Optical microscopy is a prominent tool in biomedical research and especially in the study of membrane biophysics. The plasma membrane, apart from representing the natural barrier between living organisms and the external world, is also the place where numerous phenomena, essential to the cell survival, take place, such as cellular signalling. While it is certainly important to understand the biochemistry of these interactions, it only provides a static picture of the molecular events responsible for biological activities. For example, it has been shown that in T-cell signalling, the tyrosine phosphatase CD45 is unable to access the contact zone between the immune cell and the target cell, as it is sterically excluded due to its large extracellular size [1]. Therefore, it is also necessary to gain insights into the role that lateral mobility plays in the regulation of events happening on the cellular membrane.

One of the long-standing problems in the study of lateral mobility on cellular membranes involves the role played by its structural organization. Indeed, it has now been known for a long time that the cell membrane is not just a disordered bilayer of lipids, but a dynamic landscape where molecules are constantly interacting depending on their physicochemical properties. Instead, it possesses its own transient structure, for example, in the form of lipid nanodomains, or “rafts” (Fig. 1.1), which can limit or alter the lateral mobility of membrane molecules [2, 3, 4]. These structures are usually presented as local clusters of saturated lipids, rich in cholesterol [5]. Lipid nanodomains have been an oftentimes controversial topic of great interest, since it has been hypothesized [6, 7], and demonstrated [5, 8, 9] that phase separation of certain molecules in these nanodomains naturally occurs, and appears to play a functional role *in vivo*. Lipid rafts are also thought to be responsible for the heterogeneous diffusion of particles on the plasma membrane. Raft-associated components, in fact, are thought

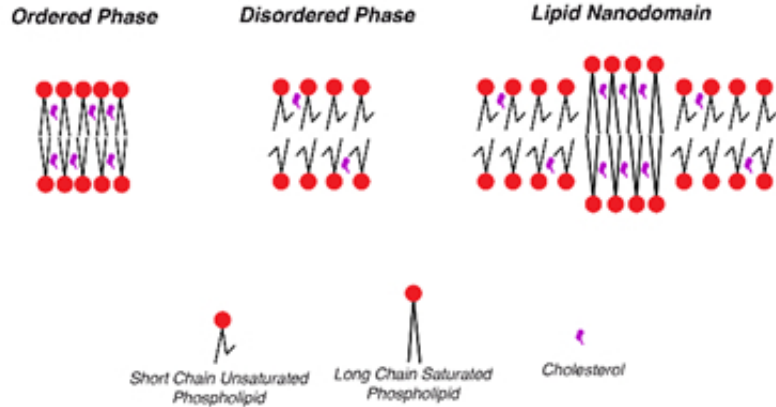


Figure 1.1: A proposed model for lipid raft composition.

to undergo transient confinements during their diffusion [6, 10, 11]. Nevertheless, the observation of such structures has proven especially challenging, owing both to their transient lifetime ($< \text{milliseconds}$) [12] and sub-diffraction size ($< 200 \text{ nm}$) [13], unless external events cause them to grow [3, 9]. Recent developments in optical microscopy, however, have helped filling this gap. In particular, techniques that managed to reach beyond Abbe's diffraction limit, have allowed to access nanoscopic levels of detail, even when investigating the living matter [8, 12, 14, 15]. The methods that have been thus far the most successful in accessing the spatial and temporal scales necessary for successfully investigating complex membrane dynamics, both in model and living membranes, have been Fluorescence Correlation Spectroscopy and Single Particle Tracking.

The basic principle behind Fluorescence Correlation Spectroscopy (FCS) is the recording of the fluctuations in the fluorescence intensity from an observation spot. Since these are mainly due to the diffusion of fluorescent molecules in and out of the observation spot, such fluctuations contain the information regarding the dynamics of the fluorescent particles present in the sample [16, 17, 18]. The derivation and interpretation of the autocorrelation function of the fluctuating signal over time makes it possible to extract parameters such as the average transit time and, consequently, the diffusion coefficient of the molecules diffusing in and out of the observation area [19, 20], while also separating them from the photophysics (bleaching, blinking) of the fluorescent tags. FCS experiments can nowadays be routinely performed on any confocal microscope with minor technical adjustments, measuring fluctuations with sampling frequencies in the tens of kHz to MHz range [21]. However, this kind of measurement would still be diffraction-limited, thus missing details and dynamics happening at smaller scales. In addition, such measurements are conventionally

limited to single-spot observations, missing spatial information on mobility. These aspects have been improved over the years through technological developments. In order to access smaller scales, the conjugation of FCS to super-resolution Stimulation Emission Depletion (STED) microscopy has been devised [22, 23, 12, 14], an approach that has allowed to reveal new insights in the nanoscale diffusion of phospholipids on living cell membranes [24, 25, 26], and even on nanoscopic objects such as viral particles [27]. Furthermore, the observation spot (conventional or STED-super resolved) can be scanned (in a line or a circle) across the sample, to improve the spatial sampling of the technique [28, 29, 30, 31, 32]. A novel, recent development, dubbed LIESS-FCS, manages to effectively combine the two approaches to achieve quasi-simultaneous sampling of super-resolved and conventional FCS data [33].

However, while FCS can provide a high level of detail on the dynamics of molecules in spots, or even limited region, it nevertheless loses the ability to produce images. In addition to this, although FCS is obviously a single-molecule sensitive technique, it may still miss some details of molecular diffusion, such as particles changing from one diffusion mode to another [12]. For this reason, the need arises to associate FCS data to a technique that can access this kind of information. Interferometric Scattering (iSCAT) microscopy-based Single Particle Tracking (SPT) measurements can be a good option, as it can achieve kHz-range sampling rates, with estimated nanometer localization precision [34]. In my DPhil project, I have explored the feasibility of using iSCAT for such experiments. In the following section I will describe the principles behind SPT and iSCAT, as an introduction to this work.

1.2 Single Particle Tracking

1.2.1 Brief Review on Single Particle Tracking

The use of Single Particle Tracking (SPT) dates back to the beginning of last century, when Perrin and his collaborators used it for the first time. The Zeiss Ultramicroscope was used for these measurements, in order to test Einstein's theory of Brownian motion and to provide an experimental estimate for Avogadro's number [35]. This experiment, which was realized with rudimental means (stopwatch, graph paper), and a time interval of 30 sec, was to be followed a few decades later by the first studies of diffusion on living cell membranes. Amongst these, we mention the tracking and analyzing the Brownian motion of micrometer-large particles on the surface of migrating human fibroblasts [36, 37]. Successive technological advances, from the '80s onwards, have made it possible to further develop the technique, both in sampling rates and

by reducing the size of the tags employed. A study by Barak and Webb, tracking fluorescently-tagged low density lipoprotein (LDL) bound to its receptor on the surface of human fibroblasts, was the first example of SPT in fluorescence microscopy [38]. So-called Nanovid Microscopy, a differential interference contrast (DIC)-based microscopy technique, was the first example of a technique developed to show observation times below a second, reaching 500 msec time resolution, while adopting 40 nm colloidal gold nanoparticles to visualize molecular diffusion in cell cytoplasm [39] and transferrin receptors on the surface of carcinoma cells [40]. The use of gold nanoparticle probes has become increasingly relevant in SPT studies on membrane diffusion since it has been possible to functionalize their surface with reactive moieties leading to molecule-specific targeting. The simultaneous development of increasingly sensitive cameras has made it possible to push the available sampling rates up to 50kHz, while claiming localization accuracies down to few tens of nanometers. This has opened possibility to uncover new insights in the diffusion of particles on the surface of epithelial cells, such as nanoscopic transient confinements [41, 42, 43, 6].

More recently, Interferometric Scattering Microscopy [44] and closely related Coherent Brightfield (COBRI) microscopy [45] have allowed to gather new insights into SPT on artificial membranes [46, 47], and living cell membrane [48, 49, 50]. These insights will be covered more extensively later in this Chapter. Briefly, the novelty introduced by these techniques is represented by the fact that they allowed an all-optical detection of biological molecules, specifically, single proteins, without the use of an external reporter in model membrane, and in principle allow label free detection and tracking of biological molecules, with nanometer localization accuracy [34]. Nevertheless, the use of pure label free scattering detection on the living cell surface is difficult due to the contemporary presence of a large number of competing scatterers.

Conversely, more recent advances in Single Particle Tracking through fluorescence microscopy, while not able to access the same sampling rates available to scattering detection, have accomplished tracking of smaller tags at kHz sampling rates to study the two-dimensional diffusion of lipid molecules on plasma membranes. Notable examples are the tracking of single nanometer-sized quantum dots bound to membrane proteins and single phospholipids up to 1.8 kHz [11], and single cholesterol molecules tagged with a fluorescent dye up to 2 kHz [51].

1.2.2 Probes for Single Particle Tracking

The necessity of introducing a label is a constant in SPT [52], and the accurate choice of the most appropriate one poses non-trivial challenges in the experiment

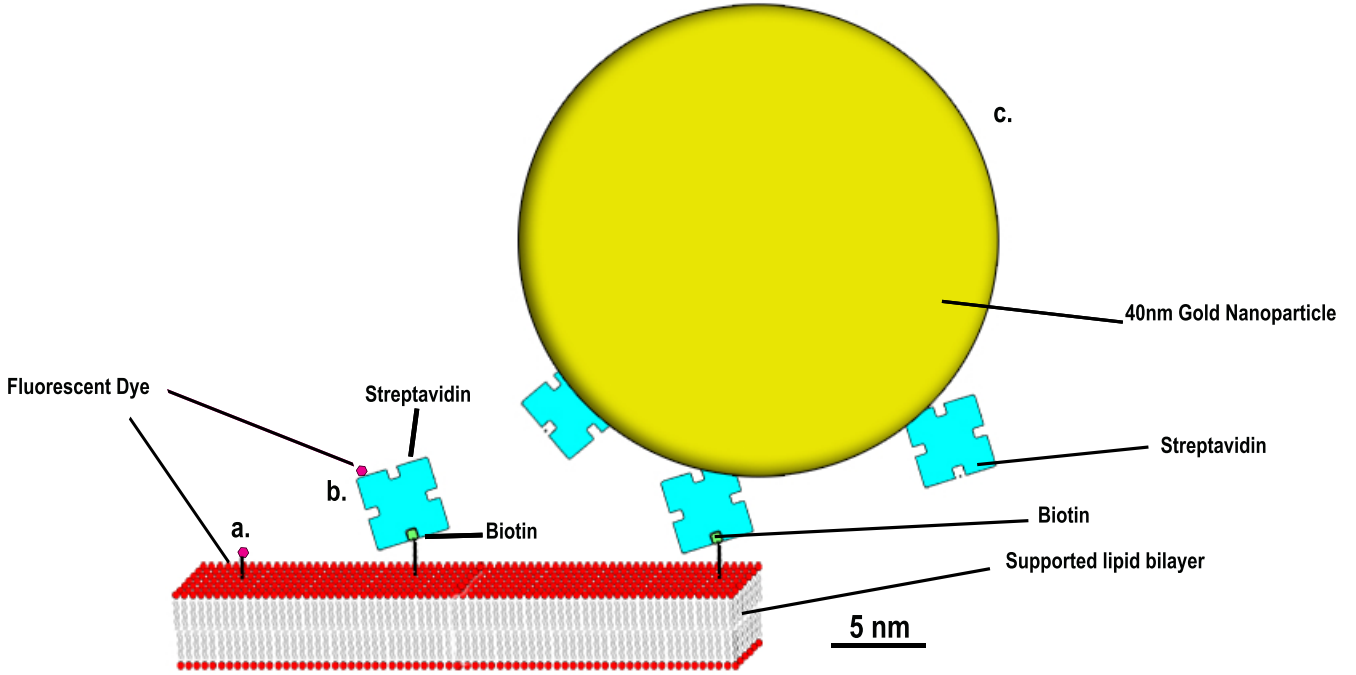


Figure 1.2: Size Comparison of some of the tags used in this work with respect to a model membrane. a.: phospholipid directly tagged with a fluorescent dye. b.: a fluorescent dye-tagged streptavidin molecule bound to a biotinylated lipid in the bilayer. c.: a streptavidin-coated gold nanoparticle binds biotinylated lipids in the bilayer, and is prevalently used in scattering-based Single Particle Tracking.

design. In choosing the appropriate probe for a SPT experiment, the way the signal is generated, in order to visualize the sample, needs to be taken in consideration. SPT mainly makes use either of fluorescence or scattering to detect moving particles [38, 39], and each of these methods carries its unique requirements. Performing successful SPT experiments generally requires the target molecule to be somehow brighter than the surrounding environment, in the case of scattering-based detection, or to be luminescent, in the case of fluorescence detection. In fact, the labels' optical characteristics have a direct influence on the choice of parameters, such as sampling frequency, observation duration, and localization accuracy. The brighter the probe, the more accurate its localization will be, and thus faster sampling rate could be achieved. At the same time, the photostability of the probe is the main requirement for a longer total observation time [53]. According to the terminology adopted by Clausen and Lagerholm ([53]), probes for SPT have a “specificity module”, and a “label module”. The former specifically binds to the molecule of interest while the latter serves to make the molecule identifiable at the detection.

Many different specificity modules have been employed over the years in biomed-

cal research making use of immunolabelling (antibodies, primary-secondary antibody design, antibody fragments, nanobodies) [54], but also employing streptavidin, due to its strong binding affinity to biotin [10, 55, 56], or cholera toxin subunit B (CTxB), due to its binding affinity for GM1 [46, 57]. The main difference between these specificity modules is that some of them, such as all species of antibodies or CTxB, have naturally occurring targets, whereas others, such as streptavidin, require the insertion of a target molecule in the sample. This last case sometimes requires non-trivial intrinsic modifications in the sample, such as genetic modification of cells or organisms [58], and should therefore be used with caution to avoid falsifying the final results. Such is also the case for Green Fluorescent Protein (GFP) and related proteins, which are attached to a target protein by amino acid sequence fusion. This last method, due to its intrinsic specificity, has been used successfully in biomedical research, but has the caveat that attaching a ~ 27 kDa protein to another could alter some of its properties, both physicochemical (translational and rotational diffusion, hydrophobicity) and physiological (biological function, binding affinities) (e.g., [59, 60]).

The choice of label module is radically different in the case of fluorescence or scattering detection. However, it is always preferable to employ the smallest possible tag, in order to reduce detrimental effects due to the size of the reporter. In fact, it is intuitive to deduce that the sheer size of a large reporter, such as large gold nanoparticles (40 nm) or quantum dots, would be enough to alter the diffusion characteristics of a tagged molecule [7]. This can indeed be a problem for three-dimensional motion studies, as can be deduced from the Stokes-Einstein Diffusion coefficient formula, or in cases where the size of a molecule directly influences its ability to participate in physiological processes [53]. Size-dependent hydrodynamics and crosslinking potential can also affect diffusion on lipid membranes. This effects can be considered not significant if we think of the molecular diffusion in terms of the Saffman-Delbrück (SD) theory, [61, 62], which states that membrane viscosity is the most influential factor in the determination of the diffusion coefficient. This theory is, however, only valid for objects embedded in a free standing membrane, with liquid bulk above and below it. Instead, SPT experiments on molecular mobility on membranes often involve the use of Supported Lipid Bilayer, with reporters binding the target molecules from above, and diffusing on top of the membrane. The geometrical restriction of diffusing near a “wall”, the breaking of the symmetry required by the SD theory, and the possibility of multiple bindings to the same tag, cause a slow down of the diffusion rate for molecules tagged with large, crosslinking labels [63, 64, 65]. Nevertheless, many studies based on scattering detection currently employ gold nanoparticle tags,

owing to the unrivalled scattering contrast obtainable through them, making it possible to reach sampling frequencies in the order of magnitude of 10 kHz with reported localization precisions in the nanometer range [6, 10, 46, 66].

Another detrimental effect is the crosslinking of several target molecules by the same tag. Many of the specificity modules reported above are characterized by some degree of crosslinking (the majority of antibodies are bivalent, CTxB can bind up to five GM1 particles, streptavidin binds up to four biotin units). Additionally gold nanoparticles and quantum dots are often functionalized stochastically, so that there is very little control over the stoichiometry of the final tag to the target molecules [54, 53]. While it has been shown that this should not lead to artifacts in the final detected diffusion mode, it will nevertheless affect the measurement in some way (e.g., slow-down in diffusion, transient trappings or confinements) [63, 67].

1.2.3 Basic Principles

The Single Particle Tracking (SPT) approach to studying diffusion dynamics follows a direction which may be regarded as complementary to that of FCS. In principle, SPT can be regarded as an apparently simple experimental technique. The typical SPT experiment, in fact, consists of recording a series of snapshots of a relatively large field-of-view. This passage is followed by the identification and reconstruction of the trajectories of particles, which can then be statistically analyzed [68, 69]. Nowadays, most of these technical steps are facilitated by the existence of advanced imaging hardware and sophisticated particle tracking algorithms, many of which are freely available [70, 71, 72]. Nevertheless, there are several aspects to take into consideration when applying this method, which, if overlooked, may lead to wrong conclusions, and generally poor experimental performance.

First of all, it should be always kept in mind that SPT relies on optical microscopy to detect the moving particles in the sample. Each particle generates a diffraction pattern in the final image, which is the convolution between the real image of the object, and the response function of the system, called Point Spread function (PSF). In the simplest case, the PSF of a microscope takes the form of the so-called “Airy pattern”. The central part of this pattern, commonly referred to as “Airy disc”, contains the brightest part of the total signal of the particle, ($\sim 84\%$), and can be approximated as a two-dimensional Gaussian $I \approx I_0 \exp[-(x^2 + y^2)/(2w^2)]$ [73, 74, 53]. In the last formula, I_0 is the total intensity of the incident light, and w is the “waist” of the incident beam, that is, its diameter in focus. According to the Rayleigh criterion, a useful convention, two particles are distinguishable when the center of these two

1.2. SINGLE PARTICLE TRACKING

patterns are no closer than the radius of the Airy disc, which is a quantity that depends on the physical characteristics of the microscope and the incident light [75]:

$$d_{Rayleigh} = \frac{1.22\lambda}{(NA_{cond} + NA_{obj})} \quad (1.1)$$

where λ is the wavelength of the light, NA_{cond} and NA_{obj} are the numerical apertures, respectively, of the condenser and the objective. When the incident and collected beams both pass through the microscope objective, then $NA_{cond} = NA_{obj}$. This differs from the Abbe resolution limit, which is defined as the smallest periodicity in a sample that can be detected, by about 20% [76, 77]:

$$d_{Abbe} = \frac{\lambda}{(NA_{cond} + NA_{obj})} . \quad (1.2)$$

Accurately identifying the position of the particles of interest, and thereby maximizing the localization accuracy of the technique, is the central point of SPT. This last concept is sometimes confused with spatial resolution. However, while in Localization Microscopy techniques (e.g., PALM/STORM) this parameter is actually useful to ascertain the subdiffraction resolving power of the technique [78], the same does not hold true for most microscopy techniques. We can define the localization precision as the statistical indetermination with which the particle is identified. This is affected by many factors, originating from the sample and the detection hardware itself [7]. It follows that in order to uniquely identify each particle at every time point, it is necessary for the target molecules to be sparsely distributed on the sample, in order to reduce the possibility of two particles to overlap. In fact, when these conventions are respected, computational image analysis nowadays commonly allows the localization of single particles to distances well below the diffraction limit, down to the nanometer scale [73, 74, 79]. The most common strategy to identify the position of a single particle in a microscopy image revolves around fitting a bidimensional Gaussian function to the particle image, following from the above approximation of the Airy disc. It is important to add that this is only possible if the image is sampled at spatial intervals, that is, effective detector pixel sizes smaller than the width of the PSF. While this may seem, in principle at least, an easy geometry exercise, in reality it presents a number of pitfalls due to the nature of the detection. Real detectors, in fact, are composed of pixels, on which the photons arrive at random positions. This makes each acquired frame vulnerable to such errors as shot noise

(Poisson-distributed noise), and pixelation, that is, the fragmentation of the image in a pre-defined number of cells, due to the geometry of the detector [80, 81].

Once the particle positions have been detected, it is necessary to connect them across the recorded movie (that is, in the time dimension) to construct the particle trajectory over time. A plethora of methods has been developed to deal with this issue, with various degrees of formal validation. The majority of methods relies on greedy algorithms for the minimization of the Euclidean distance between two successive detected points, which is the most straightforward approach. More sophisticated methods adopt cost functions to join and merge tracks through temporal gaps in which the particle was not detected, in an attempt to obtain longer tracks and gather more information on the diffusion mode of the particle, such as the well-known μ Track, developed by Jaqaman et al. [79].

1.2.4 Quantitative analysis of SPT trajectories

The quantitative analysis of SPT experiments revolves around the solution of the one dimensional Langevin equation derived by Einstein to describe Brownian motion [82], which was also derived by Langevin himself through a different derivation, citing the independent work of von Smoluchowski [83, 84]:

$$\frac{\delta p(x, t)}{\delta t} = D \frac{\delta^2 p(x, t)}{\delta x^2} \quad (1.3)$$

Where $p(x, t)$ is the probability density function (PDF) describing the probability of finding a Brownian-diffusing particle in position x at time t , and D is the diffusion coefficient. Assuming the particle is found at the origin at time $t = 0$, so that $p(x, 0) = \delta_{Dirac}(0)$, if the particle is diffusing in a purely random manner, the solution of the above Eq. 1.3 leads to:

$$p(x, t) = \frac{1}{\sqrt{4\pi Dt}} \exp\left(-\frac{x^2}{4Dt}\right) \quad (1.4)$$

Substituting this in Eq. 1.3, multiplying both sides by x^2 and integrating with respect to time, it is possible to obtain the equation for one-dimensional Brownian diffusion $\langle x^2(t) \rangle = 2Dt$ which in d number of dimensions takes the well renowned form:

$$\langle r^2(t) - r^2(t_0) \rangle = MSD_{real}(t) = 2dDt \quad (1.5)$$

Where MSD_{real} denotes the Mean Squared Displacement measured in ideal conditions, without the influence of noise. In this formula, we have adopted the notation

1.2. SINGLE PARTICLE TRACKING

MSD_{real} to specify that this is the true value of the Mean Squared Displacement, which is unaffected by measurement errors, the influence of which shall be highlighted in the next section.

Starting from this first relation, it is evident that one of the most immediate ways to extract quantitative information from SPT trajectories is through the study of the apparent MSD (MSD_{app}) against time. A wide variety of expressions has been derived to fit experimental MSD curves, from the simplest case of free diffusion (that is, constant D), to more sophisticated models of confined, anomalous, “hop” or compartmentalized diffusion, and others [85, 41, 86, 74, 87]. The factor that perhaps affects the most the statistical accuracy of the fitting result is the number of MSD_{app} points used to fit these models. In fact, given that real trajectories are made up of a finite number of points, the variance of each point in the curve increases with time. A generally accepted rule of thumb, introduced by Saxton [88], recommends that no more than a quarter of the total points should be used for fitting, and to use weighted least-squares fits of the data. However, given the large number of free parameters to be derived in more complicated models, those should require more data points for a more accurate fit. However, to our knowledge, no study has been undertaken in this direction so far.

1.2.5 Influence of localization errors

In imaging experiments, when confronted with static samples, it is possible to improve the localization accuracy by considering several measurements [89]. This is not possible for moving molecules, leading to necessarily larger localization errors. While this may seem like an elementary observation, nevertheless a great deal of literature of Single Particle Tracking reports the localization of immobilized particles, as a measurement for experimental performance (e.g.: [90, 85, 41, 34]). This might lead to an underestimation of this error, which, in turn, can distort the interpretation of the results. The localization imprecision contributes a positive, time-invariable term to Eq. 1.5, in the form of $2\delta_{x^2+y^2}^2$ [42], where δ represent the localization error in one direction. To simplify notation and analysis, we will henceforth assume that the localization error in the x and y directions is the same. While it can be argued that such a term, especially given the recent advancements of imaging techniques, is small and systematic, it is also worth noticing that:

1. for very fast sampling rates, and slowly diffusing particles, the observed particle could move less than the localization precision, leading to an artificial detected

subdiffusion [91];

2. the systematic nature of localization error is doubtful, since oscillations in the illumination source, vibrations in the setup, or changes in the sample (which is possible in the case of biological systems) can transiently affect the Signal-to-Noise ratio in the final image;
3. this error primarily affects the first few points of the MSD curve, which are the one that are classically recommended to be weighted more in the fitting [88].

Another significant error source is the motion blur, originating from the fact that during the finite camera integration time the diffusing particle is still diffusing. This contributes a negative term to the MSD curve, expressed by $-4dDRt_{lag}$, where d is the number of dimensions of the measurement, t_{lag} is the time between observations, and R is a parameter between 0 and 1/4 corresponding to the illumination profile during the integration time. Once again, this term primarily influences the points at the shortest time lags [92, 93, 94].

To summarize how the aforementioned errors negatively influence the analysis of results in SPT, we refer to Fig. 1.3.

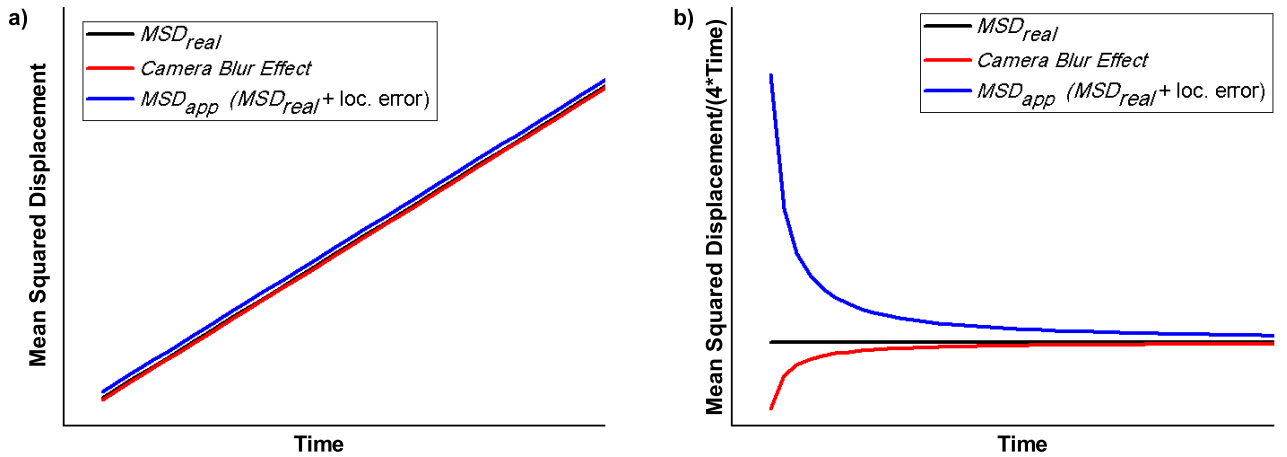


Figure 1.3: Effect of camera blurring and localization error on MSD curves. Comparison of a MSD_{real} curve, unaffected by localization noise (black) with the same values with added either camera blur (red) or localization error (blue) in the a) $MSD(t)$ vs. t linear space and b) $MSD(t)/4t$ vs. t space, where the effect of the effects of the errors are more evident. The MSD_{real} curve was calculated from Eq. 1.5 in the two-dimensional case, using $D = 1\mu m^2/sec$, and calculating the values of MSD at intervals of $t_{lag} = \delta t = 0.5msec$. The camera blur effect was calculated by subtracting a constant term $-8DRt_{lag}$, with $R = 1/6$, while the localization error is an additive constant term in the form $2\delta_{x^2+y^2}^2$, with $\delta_x = \delta_y = 20nm$.

The black line plotted in Fig.1.3a represent the behaviour of an ideal particle diffusing in pure Brownian motion on a 2D surface. As explained above, the behaviour of such particle can be explained by the relation expressed by Eq. 1.5. The red and blue lines represent the same curve, with added camera blur effect and localization error, respectively. It is evident that these three curves are almost indistinguishable, except from a small initial offset, in agreement with what explained above. However, the difference between these three expressions becomes much more evident when switching to a Mean Squared Displacement/ $4t$ vs. t representation, visualized in Fig. 1.3b. Here, the deviation of the curves where the noise has been added (red and blue) from the ideal case (black) is very evident, and it is necessary to adress it in most cases.

The example shown here, for a freely diffusing particle, is indeed very simple, as the influence of these errors is easy to discern from how the particle should diffuse in time. However, in more exotic diffusion modes (confined diffusion, “hop” diffusion), this might be underestimated or misinterpreted, and lead to incorrect conclusions. We therefore adopted an analysis method that specifically addresses this effect, which is described at length in the Materials and Methods chapter, and was initially developed in [95].

1.3 Interferometric Scattering Microscopy

1.3.1 Theory

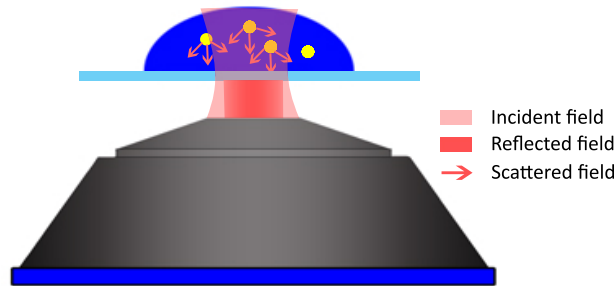


Figure 1.4: The principle of interferometric scattering detection. The incident light is partially reflected by the glass substrate supporting the sample. The component which actually illuminates the sample is scattered isotropically by the sample (here represented as spheres), and the backscattered component is collected by the objective.

The idea behind Interferometric Scattering (iSCAT) Microscopy is, in principle, very close to that of Dark Field (DF) microscopy and its derived techniques. In fact,

in both of these techniques the aim is to enhance the contrast of single scattering molecules against the background, without the use of resonant phenomena [34]. In DF microscopy, this is achieved by exclusively collecting the forward-scattered component of the incident light on the sample [96]. On the other hand, iSCAT microscopy is based on illuminating the sample with a coherent laser light source, and the contrast generation relies on the collection of a reference beam, represented by the reflection of the light by the sample support substrate, and the component of the incident light scattered by the sample in the same direction as the reflected beam (Fig. 1.4). Since both of the collected beams will still be coherent, but differ between each other in phase, they will interfere on the path to the detection camera, generating the contrast. The condition on the light source for the interference to happen is that its coherence length must be longer than the path difference originating the phase discrepancy between the two beams [97].

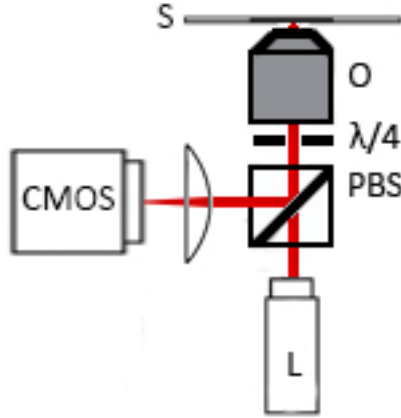


Figure 1.5: Basic scheme of a simple iSCAT microscope. The light from a laser source (L) illuminates the sample (S) through an immersion oil objective (O). The fractions of light backscattered by the sample and reflected by the immersion oil-sample substrate interface, are then reflected by a polarizing beam splitter and focused by an imaging lens (L) on the sensor of a CMOS camera. The quarter-wave plate ($\lambda/4$) ensures that the reflected and the scattered beams are correctly reflected by the Polarizing Beam Splitter (PBS) towards the CMOS sensor.

In the simplest possible configuration of an iSCAT microscope, the sample is illuminated in widefield mode by an incident laser beam (Fig. 1.5). As all iSCAT microscopes are built in inverted geometry, the components of the incident light that are then collected for imaging by the same objective are the reflection from the interface between the immersion oil and the sample substrate (usually a glass slide), and the backscattered light by the sample [98, 99, 100]. It has sometimes been argued

that iSCAT could be regarded as a digital holographic microscopy (DHM) technique, due to the similarity of the setup and the reliance on scattering for signal generation [101]. However, given the absence of an holographic reconstruction step to obtain the final image, iSCAT cannot be included in this category [45]. If we indicate the modulus of an electric field of the incident light on the sample with E_i , the reflected component can be written as:

$$E_r = rE_i e^{-i\frac{\pi}{2}} \quad (1.6)$$

where the exponent term has been introduced to account for the Gouy phase accumulation due to the reflection of a tightly focused Gaussian beam [34], while r is a real term accounting for the reflectivity of the oil-glass substrate interface. On the other side, the scattered component of light can be written as:

$$E_s = s(\lambda)E_i = |s(\lambda)|e^{i\phi(\lambda)}E_i \quad (1.7)$$

where s is the complex scattering amplitude, and $\phi(\lambda)$ is the phase of the scattered beam. For the sake of simplicity, we will in this instance restrict ourselves to the case of standard Mie theory, which holds in the case of scattering by spherical bodies of diameter d where $d \ll \lambda$, where λ is the wavelength of incident light [102]. While the quantity s is dependent on the wavelength of light λ , we will drop this dependency in the following to simplify notation, which is justifiable given the fact that the microscopy observations are done at a constant wavelength. With this assumption, which is actually valid for all of the tags used in this study, the quantity s can be expressed by:

$$s = a\epsilon_{medium} \frac{\pi d^3}{2\lambda^2} \frac{\epsilon_{particle} - \epsilon_{medium}}{\epsilon_{particle} + 2\epsilon_{medium}} \quad (1.8)$$

where a is a factor taking into account the detection efficiency, d is the diameter of the particle, and ϵ terms are the complex dielectric constants of particle and medium. The signal intensity detected by the camera can then be expressed as, given 1.6 and 1.7 [98]:

$$I_{detected} = |E_r + E_s|^2 = |E_i|^2 \{r^2 + |s|^2 - 2r|s|\sin\phi\} \approx |E_i|^2 \{r^2 - 2r|s|\sin\phi\} \quad (1.9)$$

where the term in $|s|^2$ is negligible with respect to the r^2 and the $2r|s|\sin\phi$ terms, especially in the case of very small scattering particles. The advantage of using interferometric scattering detection, collecting both the reflected and scattered component,

rather than relying on pure scattering, can be deduced from this last equation, and 1.8. In fact, in 1.9 the contrast of the scatterers is mainly due to the term in $|s|$, which scales like d^3/λ^2 (Eq. 1.8). On the other hand, the purely scattering $|s|^2$ term, instead scales like $(d^3/\lambda^2)^2$. In the Mie scattering approximation ($D \ll \lambda$), this means that the same tag, or molecule, produces much more contrast in interferometric scattering detection than in techniques where the reflected, or background, component of light is discarded. In practical terms, this means that not only similar sized particles generate a much higher contrast in iSCAT compared to dark-field techniques, which rely on the $|s|^2$ term alone, but also that the size threshold for detection becomes much smaller. In fact, it has been shown that gold nanoparticles as small as 5 nm [44] and even single unlabelled proteins [47] can be detected and tracked using this technique.

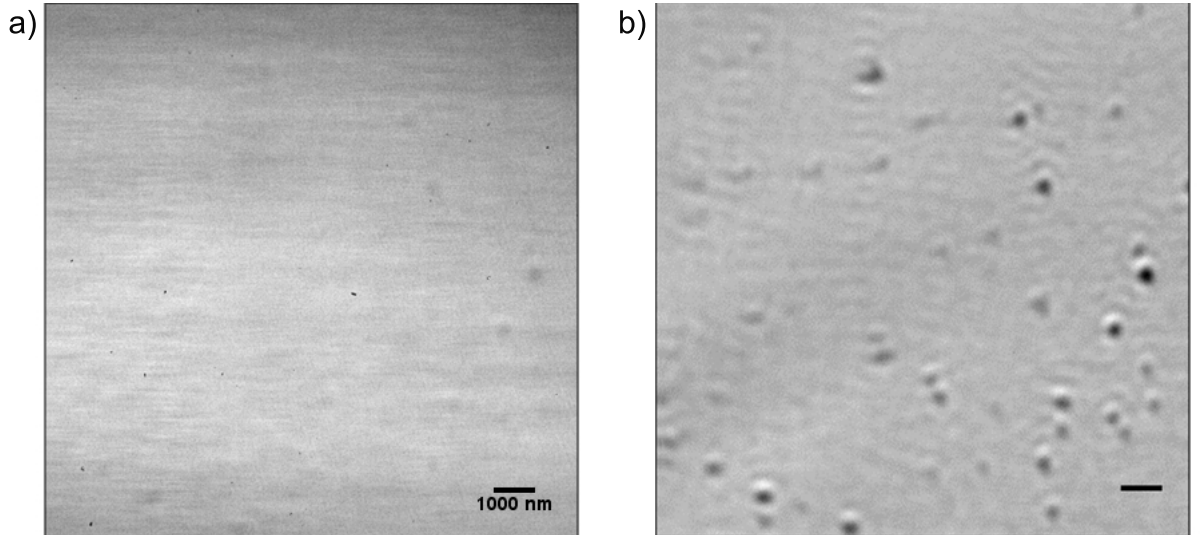


Figure 1.6: Example of an iSCAT microscope-recorded frame before (a) and after (b) the flat-fielding operation

However, all these calculations are valid not only for the scattering tags or objects of interest present in the sample, but also for any other scatterers present in the setup, at the sample support surface (e.g., roughness in the glass surface) and in the sample itself. Fortunately, the contributions of spurious reflections from the optical components are overwhelmed by the reflection at the sample support interface. As for other "static" reflections, independent of the sample, they can be easily filtered out by the so-called "flat fielding" operation. This is done by collecting a fixed number of images, moving the sample, and adopting the pixel-wise temporal median filter of this ensemble as representative of the undesired static background scattering. The pixel-wise division of the movie of interest by this median filter (also called "flat

field”) produces a movie in which the molecules of interest stand out against the background [34, 103]. Additionally, when a sample presents a significant amount of immobile particles, it is possible to also subtract the temporal average of the recorded movie, or a part thereof, to highlight only the moving fraction of the sample [104].

It is also necessary to point out, for the sake of completeness, that iSCAT microscopy has a natural way of being converted to a three-dimensional tracking technique [99, 105]. In fact, the dependence of the detected intensity (Equation 1.9) from the sine of a phase ϕ implies the possibility that the contrast of the particle may change with this quantity. The phase reported in the above equation, in reality, is a superposition of three main effects: the Gouy phase accumulation (also mentioned above), a component due to the electrostatic properties of the scattering particle (which can be calculated explicitly) , and a term which is due to the distance between the reflective interface, responsible for E_r , and the scattering particle [34, 105]. An approximate equation for the phase ϕ in function of the interface-particle distance z may be written as:

$$\phi(z) = \phi_{Gouy} + \phi_{particle} + \frac{4\pi\eta z}{\lambda} \quad (1.10)$$

where η is the refractive index of the sample medium and λ is the illumination light wavelength. However, the implementation of this method was considered beyond the scope of this study, as the main interest lied in tracking particles on flat surfaces, which only requires two-dimensional imaging.

1.3.2 Main Advantages of iSCAT microscopy

The practical advantages of iSCAT microscopy lie mainly in the inherent simplicity of the technique. First of all, while ensuring superior levels of contrast, as illustrated above, compared to other scattering-based techniques, it does not rely on any resonant effects (e.g., Raman scattering) thus avoiding the use of sophisticated supports for sample preparation [106]. Although it is still necessary to build an iSCAT-capable setup from its basic components, as we will illustrate in Chapter 2, this process has been more or less standardized. Protocols for iSCAT microscopy implementation have been published with extensive detail, for example, in [97]. In the very same work it is also outlined how it is possible to combine iSCAT with Total Internal Reflection Fluorescence (TIRF) imaging, which is yet another advantage of the technique. This combination offers the possibility of regaining some specificity in scattering measurements, and comparing iSCAT results with those obtained through a well established

imaging technique such as TIRF. While this aspect has yet to be fully explored, it has already proven useful in many of the measurements described in this work.

On the experimental side, relying on scattering carries several advantages. First of all, the SNR is effectively independent of sample features that are different from the size of the particle itself. After eliminating spurious scattering features through flat-fielding (Section 1.3.1), the residual sources of noise are in principle limited to background fluctuations. In the ideal case of a purely shot-noise limited experiment the fluctuations are proportional to $N^{1/2}$, where N is the number of photons detected by the camera. As for the signal, since iSCAT detects dips of signal on a white background, the contrast of the particle can be estimated as the ratio between the intensity of the light scattered by the particles in the sample $I_{particle}$ and the background intensity $I_{background}$:

$$\text{contrast} = \frac{I_{particle}}{I_{background}} = 1 - \frac{2r|s|\sin\phi}{r^2} \quad (1.11)$$

Since $N \propto |E_i|^2 r^2$, then the right side of Equation 1.9 becomes $I_{detected} = \text{signal} = N \cdot \text{contrast}$ and therefore, the SNR can be written as:

$$SNR = \frac{\text{iSCAT signal}}{\text{background fluctuations}} = \text{contrast} \cdot \sqrt{N} \quad (1.12)$$

in shot noise-limited regime. This implies that in iSCAT microscopy, it should be possible to tune the sensitivity of the technique simply by changing the number of incident photons N , which is a controllable quantity [34, 44, 98]. While this would also imply that the static localization precision of any iSCAT setup could reach unprecedented levels, this value eventually plateaus due to the physical limitations of the setups (spurious reflections of the optical components, stage vibrations) and non-homogeneous background scattering [107, 108]. Nevertheless, the calculation of the localization precision for static particles in iSCAT always produces values of few nanometers, as reported from many sources [109, 110, 104, 105], and in this work (see Results). Moreover, since the SNR in the final iSCAT movie only depends on the characteristics of the sample and the number of incident photons (Equations 1.11 and 1.12), it immediately follows that localization precision and sampling rate are independent of each other. This means that the sampling rate could in theory be increased indefinitely by increasing the incident laser power while keeping the signal yield and thus SNR constant. The only limitations, in this case, would be the characteristics of the detector.

Another positive consequence of the reliance on light scattering for detection is the possibility of having potentially indefinitely long observation times, as the technique is not affected by phenomena such as photobleaching or photoblinking. Additionally, phototoxicity can be limited by choosing appropriate conditions. iSCAT is also a potentially label-free technique. In fact, if a particle has a refractive index that differs from that of the medium, it will scatter light and be in principle always detectable. However, this ability to scatter light is obviously not exclusive to the particles of interest, but to everything that is present in the region of interest. This lack of specificity is one of the main obstacles that need to be overcome in iSCAT detection.

1.3.3 Application of iSCAT microscopy to Life Sciences

iSCAT Microscopy is a relatively recent technique, with the first examples of experiments dating back to 2004-2006, when it was first used to detect small gold nanoparticles close to glass substrates [44, 98]. Since then, this method has been refined and it was then applied to biological samples. A notable study used iSCAT to detect the tumbling motion and docking of fluorescent quantum-dot labeled viral particles on GM1 receptor-enriched supported lipid bilayers. The presence of the fluorescent quantum dot, while not significantly contributing to the scattering signal, due to the larger size of the virus, is used to simultaneously track the particle using fluorescence microscopy. Several other studies have been published since then demonstrating the applicability of iSCAT microscopy to several purposes, including formation of lipid bilayers [111], binding reactions [104], molecular sensing [112] and even “mass imaging” [113], in which the dependence of scattering contrast to the dimension of the scattering particle is used to estimate the particle’s weight. In this work, however, we concentrated on the application of iSCAT to Single Particle Tracking (SPT). The advantages of simultaneous excellent signal-to-noise ratio, extended observation times and fast sampling rates make this technique an ideal candidate for SPT studies on model membranes and cell membranes *in vitro*.

One of the most common strategies to detect biological molecules in scattering based techniques is via functionalized gold nanoparticles [41, 85, 114, 115]. The adoption of these tags in membrane diffusion studies is justified by the fact that the effect of increased particle size should be minimal compared to that of the viscosity of the membrane itself, at least according to standard Saffman-Delbrück theory [61, 116]. This strategy has been successfully applied to iSCAT-based Single Particle Tracking, for instance, in detecting the peculiar dynamics of different kinds of lipids on model

membranes [46, 57, 117] at unparalleled sampling rates (1 – 50 kHz) and localization precisions (few nanometers) commonly inaccessible to most SPT measurements. These studies highlight, in particular, how lipid-bound particles undergo stochastic nanoscopic and transient confinements in their otherwise unrestrained random movements on model membranes. These confinements would not have been detected with a less sensitive technique, making iSCAT an ideal tool to discern some of the finer details of molecular diffusion. Another application involved detection of myosin structural dynamics while “walking” on actin filaments *in vitro*, with results comparable to those obtained via single molecule fluorescence methods [118, 119]. In addition to these, the first study of *in vivo* tracking of scattering-enhancing labels through iSCAT has been published very recently, showing the compartmentalized diffusion of some membrane proteins on human cell membranes [120]. This experiment, targeting GPI-anchored proteins is a step in a previously unexplored direction for iSCAT SPT, that could lead to exciting results in the near future.

The potential of iSCAT as a label-free tracking technique, expressed in the previous paragraphs, has also been employed with success, showing potential to be an important tool in biomedical research. Through background subtraction, and by filtering out static features, as described before, it has been shown that tracking single proteins moving in two dimension is possible. The overall performance of this label-free iSCAT experiments, expressed in terms of estimated localization precision and attainable sampling rates, was only slightly inferior to those obtained with the use of scattering-enhancing labels. This kind of measurement has been applied successfully, in particular, to the tracking of unlabelled proteins, and specifically of membrane and globular proteins. In fact, since iSCAT was already shown to be capable of visualizing single quantum dots (~ 5 nm) [44], the extension of the same experiments to single proteins is quite straightforward [104]. The unique advantage of label-free iSCAT is represented by the possibility of visualizing unperturbed biological phenomena, which, at the single molecular level, is unachievable for most optical microscopy techniques. Tracking of unlabelled myosin on actin filaments reconstituted on glass supports, for example, has been realized with 5 nm localization precision and 2.5 kHz sampling rate [47]. Some results of unlabelled protein tracking on glass surfaces have also been published [117]. The detection of the formation and dissolution of lipid nanodomains at two membrane interfaces was also realized since highly ordered domains had a different refractive index and thus scattering characteristic than the surrounding more fluid environment [121]. All these example can be seen as testimony to the seemingly

boundless capacity of iSCAT to discern previously inaccessible details of membrane dynamics.

Another relevant development is Coherent Brightfield Microscopy (COBRI). COBRI can be considered as a close relative of iSCAT, the main difference being the illumination mode: while iSCAT operates in epi-illumination, and collects the backscattered and backreflected components of the incident light, COBRI is based on transillumination, thus collecting the transmitted light and the forward-scattered light from the sample [45]. While this method of illumination does not offer, in theory, any advantage in the sensitivity of the technique, it has been reported that when imaging living cells the iSCAT signal appears dominated by the reflections from the basal membrane of the cells. This is not the case for COBRI, which instead collects the light reflected from the top membrane of the cells. Thus, COBRI allows easier measurements of phenomena such as intracellular directed motion with microsecond time resolution [50], and early stage viral infection [49].

1.4 Objectives of the work

Given its favourable characteristics and its natural employability as a Single Particle Tracking technique, iSCAT has found a natural application in the field of Life Sciences. The camera-limited temporal resolution, affordability of the setup and label-free capabilities can offer a so far unprecedented level of detail. However, since the inception of this technique [44, 98, 99] is relatively recent, we believe that its potential has yet to be fully explored. Even though previously cited work has already ventured into imaging [122] and, more interestingly, tracking of molecules on living membranes [120], at the time of writing we are still in the early stages of this very promising enterprise. The general guideline of this project has therefore been to bridge the gap between technique development and the necessities of contemporary Life Science research. Many different approaches and experiments have been tested throughout the time spent on it, and we here report the most successful and insightful ones. The decision to apply iSCAT to the study of model and living membrane dynamics, especially lipid diffusion, has been taken to avoid incurring in the aforementioned contrast effects when applying the technique to three-dimensional problems, but most importantly in the effort of gathering new insights both on membrane biophysics, and on the analysis of Single Particle Tracking data.

First of all, the use of usually employed large (40nm) gold nanoparticle tags for SPT was validated. These tags are favourable in iSCAT due to their large scatter-

ing cross-section and signal. However, we here present a detailed study on possible tag-induced crosslinking effects and hydrodynamic hindrances, uniquely using complementary iSCAT and STED-FCS experiments. We further investigated the effect of crosslinking on diffusion characteristics by comparing the diffusion behaviour of streptavidin and an artificial monovalent variant thereof [55, 56, 58]. These effects have already been explored and modeled in [64, 65] on different protein tags.

Our iSCAT SPT experiments highlighted the need for more sophisticated analysis tools, compared to those conventionally used in SPT experiments. As we elucidated above, high sampling rate SPT measurements are very prone to bias due to the improved potential temporal resolution, which has so far been overlooked, or poorly estimated. A new analysis protocols, grounded in statistical analysis, was developed to solve this issue.

Finally, we adapted protocols for cellular labelling to iSCAT, with the objective of successfully managing to observe, acquire and analyze single phospholipid trajectories. The aim was to reveal novel details of membrane structure and organization, by comparing diffusion modes of the same lipids in cellular and model membranes..

1.5 Thesis Overview

In this Introduction (Chapter 1), we have highlighted several useful concepts for understanding the experimental procedures and the experiments, a literature review of the advancements in Single Particle Tracking and Interferometric Scattering Microscopy, and the motivation of the work.

In the Materials and Methods (Chapter 2), a comprehensive description of the Interferometric Scattering setup and an updated Single Particle Tracking analysis pipeline are presented. Furthermore, we provide general details on the experimental procedures used throughout this work.

In Chapter 3 we summarize the Single Particle Tracking experiments executed on gold nanoparticle-tagged biotinylated phospholipids diffusing on model membranes. Complementary iSCAT and STED-FCS data is highlighted, outlining the bias introduced by the large gold nanoparticle tag due to crosslinking and hydrodynamic effects related to its size [64, 65, 67]. Further, we present differences in results obtained by iSCAT recordings at slow (100Hz) and fast (2kHz) sampling rates, especially with respect to an accurate estimation of localization errors and camera blur, and applying the aforementioned analysis method.

In Chapter 4 we exploited the possibility of observing single proteins through iSCAT to isolate and observe the effect of tag crosslinking, in a manner akin to that elucidated in in [64]. Streptavidin and a monovalent variant thereof were used to label biotinylated phospholipids on a model membrane. These small (≈ 56 kDa) proteins only differ in the number of binding sites available. These results were further validated using FCS measurements. Additionally, we also present the results of the tracking of a small (≈ 2 kDa) PEG polymer attached to single phospholipids diffusing on a model membrane.

In Chapter 5 the first case of iSCAT-based SPT applied on the tracking of phospholipids on epithelial cells is reported. Gold nanoparticle-labelled saturated phospholipids were tracked at high sampling rates (2 kHz). We analyzed the data, and offer an interpretation on how the tagged lipids diffuse.

Finally, in the Conclusion chapter we offer some considerations on the viability of Interferometric Scattering Microscopy as a technique for biophysical membrane dynamics study, and some perspectives on related research directions.

Chapter 2

Materials and Methods

In this Chapter, all the technical details of the Materials and Methods adopted are presented. The building of the iSCAT/TIRF microscope setup, assembled in Prof. Christian Eggeling’s laboratory is described in detail (Section 2.1). The novelties introduced to the technique, necessary for the requirements of the experiments planned for this project, are highlighted in Section 2.1.6. Another important part of this chapter deals with the description of the method for SPT data analysis perfected over the course of the project (Section 2.2.3).

2.1 Interferometric Scattering and TIRF Microscope

The design of the purposely built Interferometric Scattering Microscope is largely based on the directions found in [46, 97]. However, I introduced several arrangements that allowed a more user-friendly and sample-oriented experimental performance devoted to biological applications. In this section, I will initially describe the three channels that make up the setup, that is, beam scanning, widefield and total internal reflection. Next, I will elucidate the workflow for image filtering and I will finally highlight the novel characteristics of this setup compared to others described in literature.

2.1.1 Beam Scanning Channel

In order to find the best compromise between fast sampling rate, relatively large field of view and image quality, this particular channel has undergone several redesigns over the course of the project, of which the two main stages are here described. In the first iteration (Figure 2.2a), the output from a 100 mW standard laser diode (OdicForce

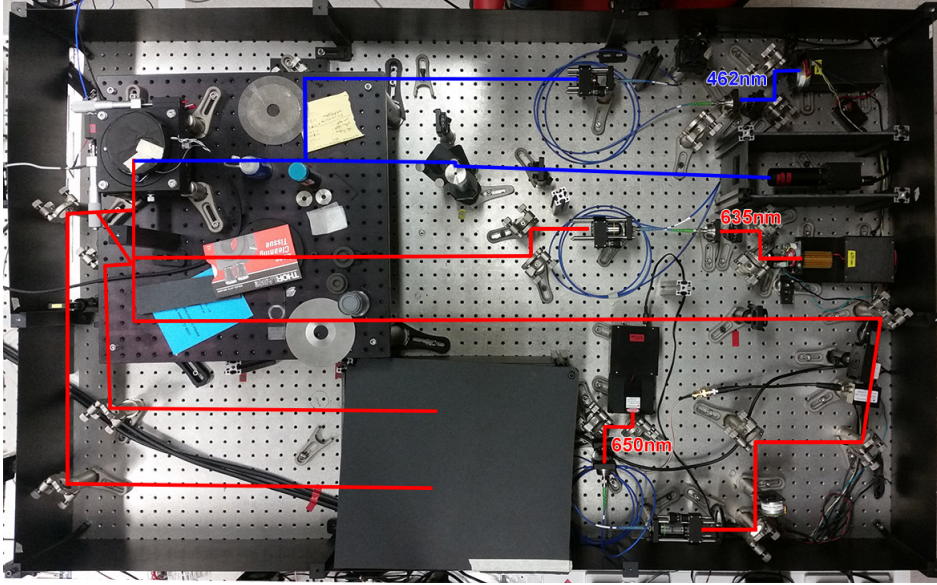


Figure 2.1: Top view of the iSCAT microscope built for the project. The overlaid lines highlight the beam paths throughout the setup in its final configuration, with a beam scanning channel (650 nm), a widefield channel (635 nm), and a fluorescence channel (462 nm)

Lasers), whose power could be regulated by a neutral density filter (Thorlabs), was expanded by a two lens system (L1 and L2, Thorlabs) to reach a beam diameter of approximately 2mm. The Acousto-Optic Deflector (AOD, Gooch&Housego) would scan the beam in the direction parallel to the optical table at a user-defined frequency. The half-wave plate positioned before the AOD ($\lambda/2$, B.Halle) ensured power maximization of the deflected mode of light passing through the deflector. The following half-wave plate ensured that the light would be p-polarized to be transmitted through the Polarizing Beam Splitter (PBS, Thorlabs). The deflected beam is then projected onto the back focal plane of the objective via a two-lens telecentric system (L3,L4, Thorlabs). A slight, not detrimental for the image quality, misalignment of the lens L4 of the telecentric system prevents spurious backreflections in the setup that would damage the optical components and create unwanted interference effects. The cylindrical lens positioned after L4 would allow, in principle, the beam to be line shaped, such that the line scanning would form a rectangular shaped field of view. The beam then went through a dichroic mirror (D, AHF analysentechnik), which couples it with the widefield illumination iSCAT channel, the aforementioned PBS, and is circularly polarised by a quarter-wave plate ($\lambda/4$, B.Halle) before being reflected into the objective O (Olympus PLAPON 60x, 1.4 NA Oil Immersion). Passing twice through the quarter-wave plate modifies the polarization of the beam so that the output is

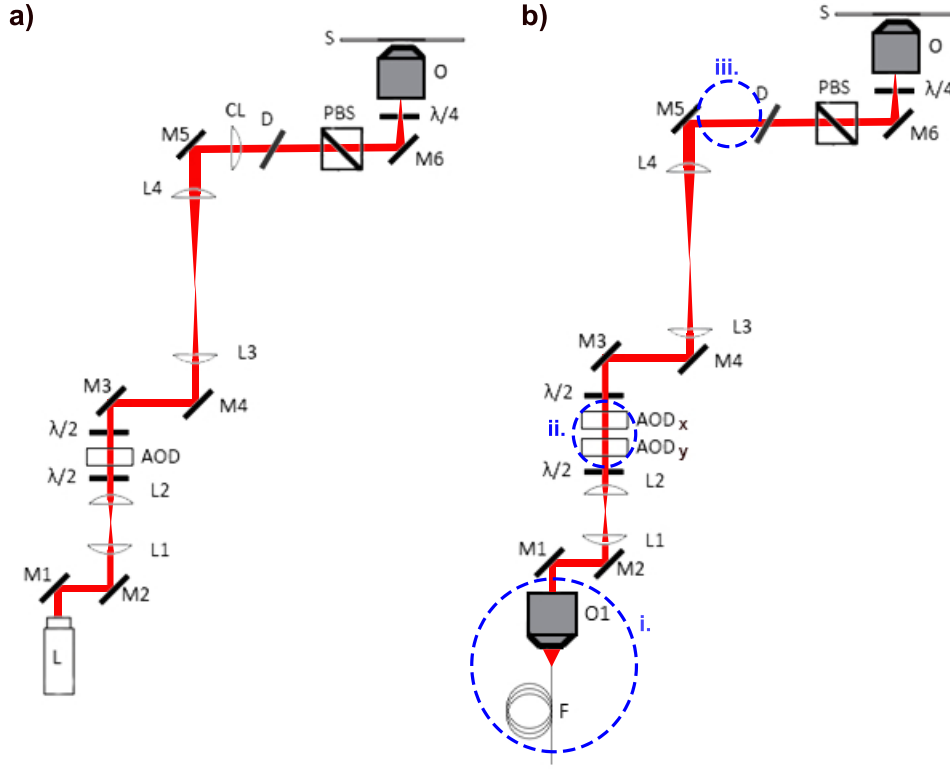


Figure 2.2: Schematics of the iSCAT beam scanning illumination channel in its first (a) and second (b) implementation. The main differences between the channels are highlighted in (b) by the dashed circles. In the first implementation, beam scanning over a plane was achieved via the use of a single AOD and a cylindrical lens. This was successively modified to a double AOD design, whose scanning was synchronized with the camera acquisition (ii.) and discarding the cylindrical lens (iii.). The laser beam was also coupled into a optical fiber (i.) to achieve a better beam profile. The M6 mirror is really a dichroic mirror, necessary for the coupling of the focus control and fluorescence channel

s-polarized, and thus reflected by the PBS on the optical path to the CMOS detector.

The main change between the two installments of the beam-scanning channel is in the removal of the cylindrical lens CL (Fig. 2.2b, iii.), and the introduction of a second AOD. This resulted in a two axes beam scanning design. While, the presence of the cylindrical lens presents a few advantages (e.g.: no need for synchronization between the AOD and the camera for acquisition), the cylindrical lens-shaped beam distributes the power not uniformly on the field of view. As a consequence, the field of view was significantly less bright on the edges of the line-shaping direction. Also, this way of scanning did not allow for full control of the field of view dimensions. The new design of the beam scanning channel is shown in Fig. 2.2b, where the main differences with the initial implementation are specifically highlighted. As anticipated above, in the new configuration two AODs would scan the beam on the x and y direction (Fig. 2.2b,

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ii.). This kind of design allows more control on the actual dimensions of the imaging field of view. The synchronization of the scanning devices and camera acquisition was made possible by the frame grabber board (National Instrument) installed in the acquisition computer. Also, the beam was coupled with 20% efficiency into an optical fiber (F, Thorlabs) and collimated using a 20x dry microscope objective (Olympus Plan Achromat, 0.4 NA) (i.), to improve the beam profile (Fig. 2.2b, i.). The power output was regulated through a variable Neutral Density filter, positioned after the collimating objective. The maximum power output was measured at 7.3 mW.

2.1.2 Widefield Channel

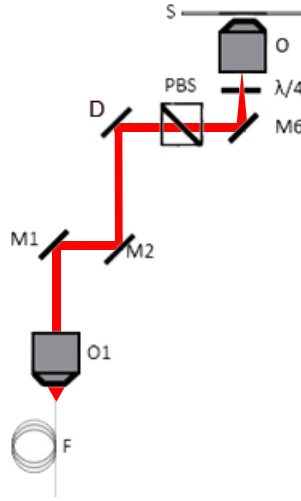


Figure 2.3: Schematics of the iSCAT widefield illumination channel. The divergent beam exiting the optical fiber F is focused onto the back aperture of the imaging objective O by the objective O1. This channel is coupled with the beam scanning channel through the dichroic mirror D (see also Fig. 2.2).

Aside from the beam scanning illumination channel, the iSCAT microscope was also equipped with a widefield illumination channel. This kind of design has been already extensively used and described in literature ([46]). In this channel, the output from a 120mW, 635nm laser diode was coupled into a fiber. The coupling efficiency was around 3%, mainly due to a rectangular shaped output mode from the laser diode, which couples very poorly with the circular section of the optical fiber (F, Thorlabs). Nevertheless, this level of power was sufficient for our purposes. Focusing of the beam into the back aperture of the objective was achieved with a dry microscope objective (Olympus Plan Achromat, 10x, 0.25NA) which collected the divergent light output from the fiber. This beam was coupled with the beam scanning channel with

the use of a dichroic mirror (Fig. 2.3,D and Fig. 2.2b, D) (AHF analysentechnik AG), and then followed its same beam path towards the imaging objective O and the detection channel. To this end, beam steering was achieved using mirrors M2 and D, while mirror M1 was placed in the setup to allow a more convenient design. The polarization needed for the beam to be compatible with the use of the PBS in the same manner as the beam scanning channel was achieved through the rotation of the output port of the optical fiber.

2.1.3 Detection

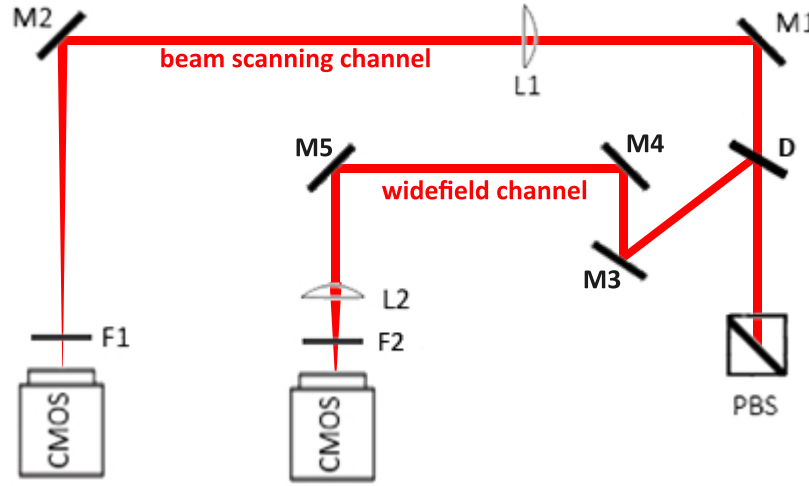


Figure 2.4: Schematics of the iSCAT detection beam paths. This channel receives the signal output from the illumination channels thanks to the polarizing beam splitter (PBS) (see also Fig. 2.2 and Fig. 2.3).

The output signal from the imaging objective, comprising the reference beam and the backscattered component from the sample, is reflected by the polarizing beam splitter (PBS) on the detection paths (Fig. 2.4), as it is polarized perpendicularly with respect to the input light. The two channels were then split by wavelength using a dichroic mirror (D, Thorlabs), which reflects light up to 638nm wavelength. However, since the widefield channel's wavelength was 635 nm, while the beam scanning channel's was 650 nm, the separation of the light operated by the mirror when positioned at 45° was suboptimal. Therefore, we opted to mount the element at a different angle, determined by measuring the power of the light transmitted and reflected by the mirror at different inclinations. Through a process of trial and error, we obtained that roughly 80% of light power was correctly reflected or transmitted, which was deemed satisfactory.

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The transmitted beam, that is, the beam scanning one, was then focused onto a CMOS camera (PhotonFocus) using a 1000 mm focal length imaging lens (L1, Thorlabs), achieving a final effective pixel size of 31.8 nm. The reflected beam, the widefield one, was then steered into the more standard right-angle geometry for ease of positioning of the 750 nm focusing lens (L2, Thorlabs). The effective pixel size was in this case of 42.4 nm. Single wavelength filters (F1 and F2, AHF analysentechnik AG) were positioned in front of each of the two channel's cameras to avoid spurious effects coming from the leaking of light from the other beam.

This iSCAT channel was built to achieve higher sampling frequencies (up to 30 kHz), with smaller fields of view ($\sim 1\mu\text{m}$ across). While some experiments were indeed carried out using this channel, their analysis proved of difficult interpretation, and are not mentioned in this work. The design of this channel is hereby reported for completeness.

2.1.4 Focus control and fluorescence channel

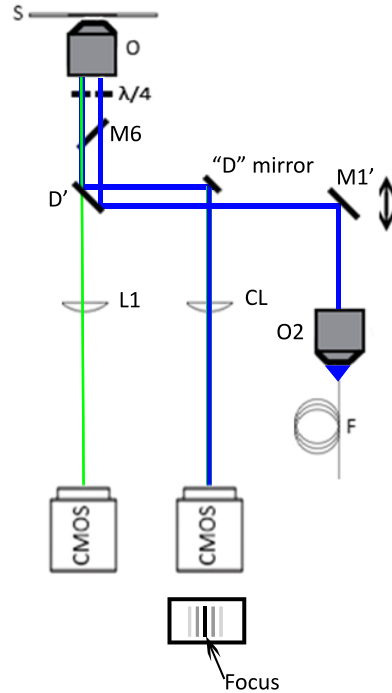


Figure 2.5: Schematics of the fluorescence and focus control channel. This channel is coupled with the iSCAT beam scanning and widefield channels through the dichroic mirror M6 (see also Fig. 2.2 and Fig. 2.3).

The output from a 100mW, 462nm laser diode coupled into an optical fiber (F, Thorlabs), was collected by a dry microscope objective (O2, Olympus Plan Achromat,

4X, 0.10 NA), which focuses the beam on the back aperture of the imaging objective O. The mirror M1' is mounted on a translation stage (Thorlabs), in order to change the the angle at which the light arrives on the sample, and achieve total reflection conditions. Beam steering was done by utilizing the M1' mirror and the dichroic mirror D'. The light output from the sample contains therefore two components: the fluorescence output, generated by suitably tagging the sample with blue-absorbing, green fluorescent dyes, and the backreflection of the incident blue light. The fluorescent component travels through the dichroic mirror D', and was focused on a CMOS camera (FLIR Machine Vision, Grasshopper3) by the lens L1 (focal length 500 nm, Thorlabs). This configuration, coupled with the camera specification, resulted in a 42.4 nm effective pixel size. The images produced by this channel were mainly used to validate the state of the sample under imaging with iSCAT, without further analysis. In order to prevent spurious signals ("leaks") to reach the camera, we put filters that blocked light at 462 nm, 650 nm and 635 nm in front of it (AHF analysentechnik AG).

The backreflection from the objective is reflected by the dichroic mirror D', but was reflected again, perpendicular to the illumination path, through a cylindrical lens (300 nm focal length, Thorlabs), and towards the focus control camera (FLIR Machine Vision, Firefly). The focus position is controlled by monitoring the displacement of the line-focused beam when the sample drifted in the Z direction [123, 97]. An active feedback loop, programmed in a LabView application (courtesy of Prof. Kukura's laboratory), used a piezo microscope objective positioner (Piezosystem Jena GmbH) to maintain the focus position over time. However, after the first experimental applications of the microscope, we did not use this focusing method further. In fact, while in most application this method achieves good focus stability over time, it was not suitable for the experiments we pursued, as with the majority of our samples it resulted in the camera-pizeo actuator feedback loop overcompensating, producing very significant oscillations in the focus position after only a few cycles. It is possible that the origin of these problem lays in the design of the sample holders we utilized, that applied pressure to the glass slides, thus generating microscopic deformations which affected its performance. Furthermore, while in most cases the focusing is controlled by a stabilized stage [97], in our implementation the focus control element acted directly on the objective, via a piezo positioner (MIPOS, Piezosystem Jena GmbH). This system, however, still ensured a good stability of the focus position for limited periods of time ($\sim$ sec), which were sufficient for our experiments.

2.1.5 Image Processing Workflow

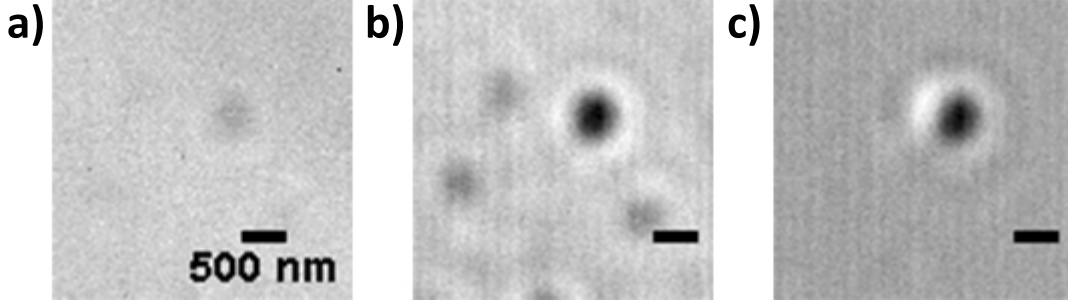


Figure 2.6: Illustration of the iSCAT image analysis workflow through imaging of a 40 nm gold nanoparticle. a) Raw movie frame as acquired by the CMOS camera on the beam scanning channel. This frame was acquired at 2 kHz sampling frequency. b) Median-filtered version of the same frame. c) Temporal average-subtracted version of the same frame.

Data acquisition for the iSCAT microscope is controlled by a LabView module (courtesy of Prof. Kukura’s group), which also manages the synchronization between the AOD beam scanning and sensor exposure. The LabView module produces output files in the form of non-interleaved *.tdms* files, a National Instruments proprietary file format, in which the movie is flattened as a singular array of 16-bit values. The median filter and the raw iSCAT moviefiles were converted to *.tdms* by a simple Matlab script, using the function “ConvertTDMS v10” (available online at <https://www.mathworks.com/matlabcentral/fileexchange/44206-converttdms-v10>).

Since iSCAT movies contain static features originating from different reflections in the sample (Fig. 2.6a), it is necessary to isolate and eliminate these contributions through a filtering operation. To this end, before the acquisition proper, 1000 frames are collected while laterally translating the focused sample. The static features are then well represented by the temporal median of these frames. By dividing the original movie by this image (flat-fielding), to which we shall refer as “median filter” in the following, it is possible to obtain a movie in which the only sources of scattering visualized are the ones originating from the sample [103, 34]. Furthermore, we subtracted from the complete movies their temporal average, in order to highlight the mobile fraction of the sample alone [104]. The aforementioned image processing operations, together with stack brightness adjustment, were executed by purposely written FIJI-ImageJ batch processing macros [71, 124, 125].

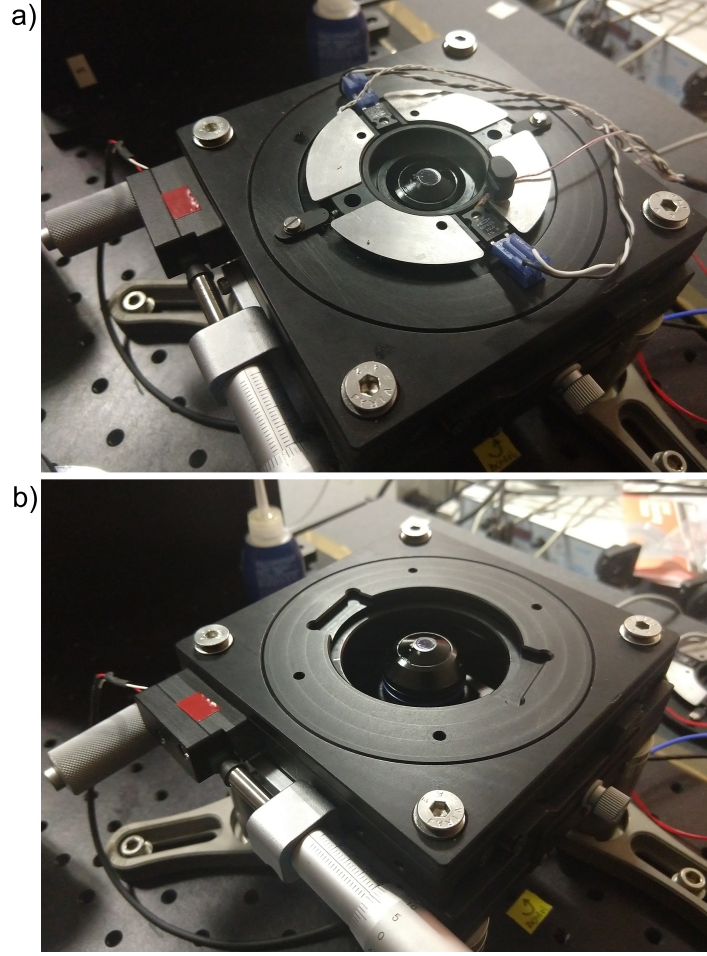


Figure 2.7: Sample stage of the iSCAT-TIRF microscope. a) The sample stage with the temperature control adapter, designed to fit $\varnothing 25$ mm and $\varnothing 18$ mm glass stainless steel sample holders. b) The glass slide adapter, especially designed to fit the 8-well μ -slide (ibidi) routinely used in Prof. Eggeling's laboratory.

2.1.6 Summary of the innovations introduced to the technique

The setup that we just described was developed and built in the same department as the Wolfson Imaging Centre, with ease of access to cell culture laboratories and state-of-the-art commercial and custom microscopes. This environment has been ideal to fully explore the applicability of the iSCAT technique to the life sciences and compare the performance of iSCAT to other established techniques (e.g., FCS, STED-FCS). Therefore, a few modifications to the workflow and the setup as it is described in literature (notably in [97]) were deemed necessary.

From the hardware point of view, the main innovation come from the design of the sample stage (Fig. 2.7). In order to increase the user-friendliness of the experiments,

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the setup was equipped with an adapter especially designed to fit a temperature control chamber (Warner Instruments), which is pictured in Fig. 2.7a. This device allows easy temperature control, and is compatible with stainless steel, water-tight sample holders, routinely used in Prof. Eggeling’s laboratory. These sample holders, which could house $\varnothing 18$ mm or $\varnothing 25$ mm cover glass slides offer several advantages when compared to the silicon gasket that are often reported to be used in iSCAT experiments (e.g., [46]) :

- (i) they are compatible with all other microscopes present in the facility, allowing the same sample to be measured with multiple techniques;
- (ii) they allowed used of standardized protocols, optimized for these supports (e.g., production of Supported Lipid Bilayers through spin-coating, cell culture);
- (iii) they are easier to use, and present fewer possibilities of user error, that could result in loss of sample;
- (iv) they could be heated without issues using the temperature control chamber, allowing cell measurements at physiological temperature;
- (v) the samples can be hydrated with larger quantities of liquid, ensuring prolonged observation periods.

Another adapter was designed and realized to use chambered $300\mu\text{l}$ 8-well slides (ibidi) (Fig. 2.7b).

Nevertheless, this choice also presents a few drawbacks. First of all, as it was already explained in Section 2.1.4, we deem this to be the cause for the malfunctioning of the focus stabilization channel. Also, a more extended sample means that finding a suitable region of interest in the sample requires more time, and displacing the sample on the stage also inevitably causes the focus to drift. The first issue was dealt with by using the piezo actuator and amplifier (Piezosystem Jena) in closed loop configuration, so that they would maintain the focus position as much as possible. This was found to be sufficient to allow observations of a few seconds of duration, which was enough for the planned experiments. The second issue is common to many experiments and commercial microscope setups, so the microscope stage was simply refocused whenever the sample drifted out of focus.

Another novel point of our configuration is the development of a workflow to convert the data from the proprietary National Instruments *.tdms* format into *.tif* files, which are readable by any computer, and easily shareable (see 2.1.5). This

conversion process also proved useful in applying well-established both freeware and commercial algorithms for single particle tracking. Using optimised image analysis software which are the standard amongst the biomedical imaging community, will facilitate the access to the technique.

2.2 Single Particle Tracking

2.2.1 Feature Identification and Tracking

The iSCAT movies recorded were analyzed using commercially available, or widely recognized feature identification and tracking algorithms. However, most of these softwares are optimized for analysis of fluorescence-based data (e.g., TIRF microscopy). Therefore, they are designed to detect bright spots over a dark background. This issue was easily solved by applying an inversion operation to the iSCAT movies.

The identification and tracking of the particles was done using two different softwares. For the tracking experiments of gold nanoparticle-tagged lipids at low sampling rate (Chapter 3), the movies were loaded in the MatLab TrackNTrace application. Sub pixel localization was achieved by fitting the spot profile with a two-dimensional Gaussian. The diffusing particles were tracked by the widely-adopted μ Track algorithm, which is implemented in the script itself [70, 72]. For higher sampling rates experiments, I adopted the Spot Detection feature in the Imaris image analysis software (Bitplane AG), which calculates the centroid of the spot for sub-pixel localization. The tracks are derived using an in-built nearest neighbour algorithm.

The only conditions that were posed on the tracks is that they should present no gaps, given that scattering-based detection is immune from phenomena such as photobleaching and blinking. This potentially reduced sample size, but also ensured that the same particle was tracked through time.

2.2.2 Data Analysis

The quantitative analysis of SPT experiments, after the centroid positions at each time frame have been extracted, starts by determining the Mean Squared Displacement of the particle as a function of the time interval between two positions:

$$MSD(t_n) = \langle (r(t) - r_0)^2 \rangle = \frac{1}{N - n - 1} \sum_{i=1}^{N-n-1} |\underline{r}(t_{i+n}) - \underline{r}(t_n)|^2 \quad (2.1)$$

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Where $\underline{r}(t)$ is the position vector of the particle centroid at time t [41, 85]. This quantity then has to be fitted to a suitable model, in order to extract useful quantities to describe its diffusive behaviour, which are based on the equation formulated by Einstein of 1D Brownian diffusion, which links MSD, diffusion coefficient (D), and time:

$$MSD(t) = \langle (x(t) - x_0)^2 \rangle = \int_{-\infty}^{+\infty} x^2 N[0, 2Dt] dx = 2Dt \quad (2.2)$$

where $x(t)$ is the position at time t , x_0 is the position of the molecule at time $t = 0$, and $N[0, 2Dt]$ is the normal distribution of mean zero, and variance $2Dt$ [126]. This formulation was later generalized to higher dimensionality problems:

$$MSD(t) = 2dDt \quad (2.3)$$

where d is the number of dimensions of the problem [35, 127]. Evolving from this, a large number of models have been developed to accurately describe more complex dynamics, for phenomena such as Brownian diffusion [35, 126, 127], confined diffusion [41, 128], hop diffusion [129], directed diffusion (“flow”) [130] and combinations thereof. Usually the identification of the kind of diffusion dynamic can be done by analyzing the shape of MSD when plotted against t [52].

A considerable issue in analyzing MSD vs t curves comes from selecting how many points to fit to the model. In fact, due to the nature of the MSD calculation, the bigger the time lag t from the beginning of the track, the less points are averaged, generating a larger statistical error on each point [85]. A generally accepted rule of thumb is to fit $MSD(t)$ point corresponding to no more than 25% of the total duration of the track, using the weighted least-squares of the data [88]. We decided to adhere to this convention, although there is no unanimous consensus over this matter. This is, however, a very critical point, since the number of points to use in a fit is perhaps the most fundamental issue of all. This is particularly true when the chosen model for fitting contains many free variables.

Apart from this issue, there are other sources of error, of purely experimental origin, that we needed to be take into account when approaching this kind of experiment. The contribution arising from the finite localization precision has been shown to contribute an additive term to the diffusion equation [42]. We therefore adopted the following model to fit our two-dimensional MSD curves:

$$MSD_{app}(t) = 4D_{app}(t)t + 2\delta_{x^2+y^2}^2 \quad (2.4)$$

which is essentially equation 2.3 with $d = 2$ and with the addition of a localization precision term. We have indicated with MSD_{app} the mean squared displacement to stress the apparent nature of the number, whereas the same quantity, unaffected by measurement errors, should be indicated as MSD_{real} . D_{app} indicates the apparent diffusion coefficient, following a very similar convention to [95]. The *app* subscript is added to take into account different diffusion modes, with different possible expressions of D .

This data analysis method, which introduces no novelty compared to what already established in literature, has been adopted to analyze the low (100Hz) sampling rate experiments from Chapter 3. In this case, a simple two-dimensional free diffusion model ($D_{app}(t) = \text{constant} = D$) was adopted to explain the diffusion of gold nanoparticles on a Supported Lipid Bilayer. The MSD values from each track were also ensemble-averaged to determine an average value for the diffusion coefficient D . High sampling rate (2kHz) experiments required a more advanced analysis approach, which we elucidate in the following.

2.2.3 Advanced Data Analysis for SPT Data

In order to properly approach the problem of analyzing single particle trajectories, it is first necessary to revise the issues linked to the localization error. The correct interpretation of this imprecision is particularly pressing in high sampling rate single particle tracking experiments. In fact, it has been shown to originate fictitious subdiffusion processes [91], which can lead to erroneous deductions regarding the diffusion mode of the particle.

The majority of literature sources (e.g., [34, 85, 90, 41]) estimate the localization precision of scattering-based single particle detection by localizing immobilized particles on clean surfaces. This method is indeed valid to evaluate the performance of the single instrument in ideal conditions. It could be considered imprecise in real experimental conditions, given that a moving particle on a highly scattering surface, like a living cell membrane, will not produce the same Signal-to-Noise ratio as the immobile counterpart. Furthermore, since real cameras have a finite integration time, each particle snapshot is unavoidably affected by motion blurring. This is a well-known effect, and it has already been studied and effectively modelled. In particular, it has been found to originate a negative contribution term, to be included in Eq.2.4, which is expressed by [92]:

$$- 4dD_{app}Rt_{lag} \quad (2.5)$$

2.2. SINGLE PARTICLE TRACKING

where t_{lag} is the exposure time of each movie frame which, in general, is equal to the time between observations in SPT. The parameter R is a term which depends on the illumination profile over the integration time, and follows $0 \leq R \leq 1/4$. In particular, it is $R = 1/6$ for full frame averaging. It is necessary to take this effect into account when adopting high framerates. As a matter of fact, this term affects more significantly the first few points especially, since it has been established that the initial part (usually, the first 25% [88]) of the MSD curves should have more weight in data fitting. It is plausible to assume that the systematic omission of this correction comes from the fact that this effect was not evident when Single Particle Tracking was first adopted, owing to larger time intervals between particle observations. In light of these considerations, we elaborated an updated approach to analyzing single particle trajectories, which is summarized in the flowchart in Fig.2.8.

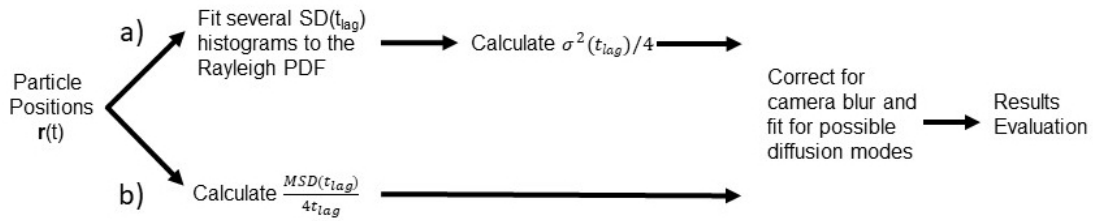


Figure 2.8: Workflow for Single Particle Tracking Analysis. More details on each passage are given in the text. Sides a) and b) are not alternative, but complementary.

The workflow starts, as usual, with the detection of the particle positions over time, and the identification of trajectories. We subsequently select the trajectories which have an arbitrary number of points, above a cutoff value. This value is different depending of experiments, but is always above 500 points (equivalent to 0.25sec at 2kHz sampling rate). Additionally, the tracks selected will not have any temporal gap.

As we would do in the canonical way of data analysis, the Squared Displacements and the Mean Squared Displacements at each time lag are calculated, according to Eq.2.1. At this point, we adopted a double approach to the data fitting, which are represented as route a) and b) in Fig. 2.8, which we shall regard not as alternative to one another, but complementary. In fact, there is no *a priori* reason to prefer one to the other. Therefore, the results obtained through both methods will be reported for each instance of application.

According to a), we collect the Squared Displacement (SD) of the trajectory for a number of specified time intervals without averaging them:

$$SD(t_n) = \{|\vec{r}(t_{i+n}) - \vec{r}(t_i)|^2\} \text{ with } i \in [1, N - n - 1] \quad (2.6)$$

we define $t_n = n * t_{lag}$ to simplify the notation, with n variable in the range:

$$n \in [1, 10] \cup \{15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100\} . \quad (2.7)$$

In order to keep the analysis times reasonable, we restricted the study of the distribution of $SD(t)$ to the points described by n . Although this choice is arbitrary, and should be extended whenever the computational time and power allows for it, we chose a subset we deemed reasonable, given that the points are all well within the first 25% of the total points in the trajectory. Also, the density of the points chosen is higher at the beginning of the set, where noise effects should be more significant, and which should be more relevant in data fitting [74, 88] (also see Introduction, Section 1.2.5). Once all the SD at the selected time points have been collected, we can generate a distribution from the values. Given the fact that the SD , at each time point, is a positive value random value, it is always possible to fit its distribution using a one-dimensional Rayleigh Probability Density Function (PDF) [131, 132], in the form:

$$P(\sqrt{SD(t_n)}, t_n) = 2\pi A \frac{SD(t_n)}{\sigma_n^2 t_n} \exp\left(-\frac{SD(t_n)}{\sigma_n^2 t_n}\right) \quad (2.8)$$

in which we stress that t_n is a constant for each fitted Rayleigh PDF. The time dependency of the formula means that the PDF becomes larger as the value of time lag t_n increases (Fig. 2.9). The parameter σ_n^2 , therefore, has the same dimensions as a Diffusion Coefficient ($[r^2]/[t]$). The relation between this parameter and the Diffusion coefficient is, in fact $\sigma_n^2 = 4D_{app}(t_n)$ [133], for a two-dimensional problem, like the ones considered in this work. By fitting the PDF in Eq. 2.8 to each distribution, we thus obtain the values for the diffusion coefficient $D(t)$ (time-dependent, in the generalized form) at each t_n , which will later be fitted to different models for diffusion. We shall refer to this method as “Step Size Distribution” in the following.

Once the values for MSD_{app} have been calculated, on side b) of the flowchart in Fig. 2.8, we reproduce the new take on the analysis of MSD vs. time, found in [95]. Once all of the $MSD(t)$ have been calculated, we divide the results by $4t$ to obtain the value for the apparent diffusion coefficient at each time point, since it can be easily derived from Eq. 2.4 that:

$$\frac{MSD_{app}(t_n)}{4t_n} = D_{app}(t_n) + \frac{\delta_{x^2+y^2}^2}{2t_n} \quad (2.9)$$

In line of principle, having the values of $\sigma_n^2/4$ and $MSD_{app}/4t_n$ should already allow us to reach the conclusion of our workflow. However, since we have no *a priori*

Fitting model:

$$P(\sqrt{SD(t_n)}, t_n) = 2\pi A \frac{SD(t_n)}{\sigma_n^2 t_n} \exp\left(-\frac{SD(t_n)}{\sigma_n^2 t_n}\right)$$

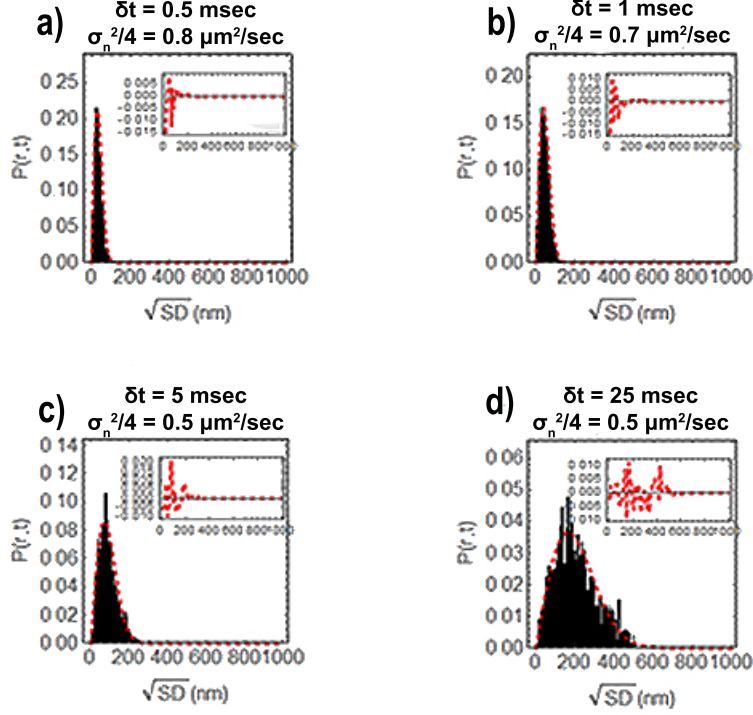


Figure 2.9: Fitting the distributions of Squared Displacements at different time points for a diffusing particle. Once the $SD(t_n)$ for different t_n have been calculated, it is possible to fit them to a Rayleigh distribution (Eq. 2.8), where the parameter σ^2 is related to the apparent diffusion coefficient. The panels show how the distribution changes when calculated at different timepoints: a) 0.5msec, b) 1.0msec, c) 5msec, d) 25msec. In black, the normalized histogram of occurrences of values of $SD(t_n)$, while the red dashed lines are the fitted Rayleigh distribution. The insets in each panel show the residuals of the fit in red.

knowledge of the experimental system under study, we cannot bar the influence of camera blurring on the measurements, expressed by Eq. 2.5. This kind of error can be especially significant when measuring the diffusion of small, fast tags which can sensibly move during one camera integration time. For what expressed before, and in Section 1.2.5 of the Introduction, it is therefore necessary to include it, for example, in the Mean Squared Displacement expression by putting together Eq. 2.4 and Eq. 2.5. The following calculations have already been presented in [95], and are here reported for clarity:

$$MSD_{app}(t_n) = 4D_{app}(t_n)t_n + 2\delta_{x^2+y^2}^2 - 8D_{app}Rt_{lag} \quad (2.10)$$

Where the time variable has been explicitated as $t = t_n$, with $n \in [1, N]$ for an N-position long trajectory, since all observations are equidistant in time. This can be rewritten as:

$$\frac{MSD_{app}(t_n)}{4t_n(1 - 2R/n)} = D_{app}(t_n) + \frac{\delta_{x^2+y^2}^2}{2t_n(1 - 2R/n)} \quad (2.11)$$

Therefore, by dividing the previously obtained $MSD_{app}(t_n)/(4t_n)$ by $(1 - 2R/n)$, it is possible to correct for camera blurring. It is necessary to substitute $R = 1/6$, which corresponds to full frame averaging, as highlighted in [92]. The last two passages can also be extended to the values for $\sigma^2(t_n)$ obtained from the Step Distribution Analysis, therefore:

$$\sigma^2(t_n) = 4D_{app}(t_n)t_n + 2\delta_{x^2+y^2}^2 - 8D_{app}Rt_{lag} \quad (2.12)$$

or

$$\frac{\sigma^2(t_n)}{4(1 - 2R/n)} = D_{app}(t_n) + \frac{\delta_{x^2+y^2}^2}{2t_n(1 - 2R/n)} \quad (2.13)$$

from which we can obtain the blur-corrected $\sigma^2/4$ values. In the remainder of the text, we shall refer to “camera-blur corrected” data to indicate the values of $\sigma^2(t_n)/4$ and $MSD_{app}(t_n)/4t_n$ which have been divided by $(1 - 2R/n)$, with $R = 1/6$, and n corresponding to the value of $t_n = nt_{lag}$. Finally, it is possible to actually fit different models for D_{app} to the blur-corrected data obtained from both analysis methods. For the sake of consistency, we used the same subset of values of $MSD_{app}(t_n)/4t_n$ as $\sigma^2(t_n)/4$, by only fitting the models to the values with n in the interval described below Eq. 2.6. We chose not to apply any prior knowledge or preconception on the fitting operation, therefore each dataset was fitted to all the chosen model. Since we used (theoretically) homogeneous model membranes, and living cell membranes, it is reasonable to attempt fitting using the following three models:

- free, unrestricted diffusion

$$D_{app}(nt_{lag}) = D \quad (2.14)$$

- confined diffusion

$$D_{app}(nt_{lag}) = D_\mu \left(\frac{\tau}{t} \right) \left[1 - \exp \left(-\frac{\tau}{t} \right) \right] \quad (2.15)$$

2.2. SINGLE PARTICLE TRACKING

- hop diffusion

$$D_{app}(nt_{lag}) = D_M + D_\mu \left(\frac{\tau}{t} \right) \left[1 - \exp \left(-\frac{\tau}{t} \right) \right] \quad (2.16)$$

The free diffusion model in Eq.2.14 is very well known, and correspond essentially to the case in which the particle moves freely, that is, the diffusion coefficient is time-independent. The model for confined diffusion is sometimes written with $D_\mu = L^2/(12t_n)$, where L^2 indicates the area in which the particle is confined, and the parameter τ found in the exponential describes the residence time within said area [129]. In the hop diffusion mode [10], a particle jumps between areas of transient confinement, with average residence time τ . This mode of motion is expressed by Eq. 2.16, which is essentially the combination of Eq. 2.14 and Eq. 2.15. Here we define a “macroscopic”, constant diffusion coefficient D_M , which is characteristic of the motion between the confinement areas, and a “microscopic” one, which the particle exhibits while transiently confined. A quick way of visualizing the differences between the different models is provided in Fig. 2.10. The free diffusion curve tends to plateau to the value of D , after the initial peak due to the localization error effect. The confined and hop diffusion start at higher values than the free diffusion model, but while the confined diffusion tends to zero for $t \gg \tau$, the hop diffusion model tends to a free diffusion, levelling off at the value of D_M .

In order to simplify notation in the remainder of the text, we will use the notation $t = t_n$ when dealing with the results of the SPT experiments. Since each of these model calculates the localization error $\delta_{x^2+y^2}^2$, it is even possible to execute a second correction, by subtracting this value to the MSD_{app} and the $\sigma^2(t)/4$, in an attempt to fit their “real” values, using the equation:

$$D(t) = \frac{\sigma^2 t - 2\delta^2}{4t} \text{ and } D(t) = \frac{MSD_{app}t - 2\delta^2}{4t} \quad (2.17)$$

The quality of the fits is then assessed through statistical means. As a reference, the reduced χ^2 for each diffusion model is calculated, although this parameter is not very useful in the case of non linear fitting. The goodness of the fits is more aptly evaluated via the calculation of the Bayesian Information Criterion for each model fit, using the formula [134]:

$$\text{BIC} = k \ln(n) + n \ln(RSS/n) \quad (2.18)$$

where n is the number of data points, RSS stands for the Residual Sum of Squares, and k is the number of parameters in the fit. The lower the value of BIC, the higher

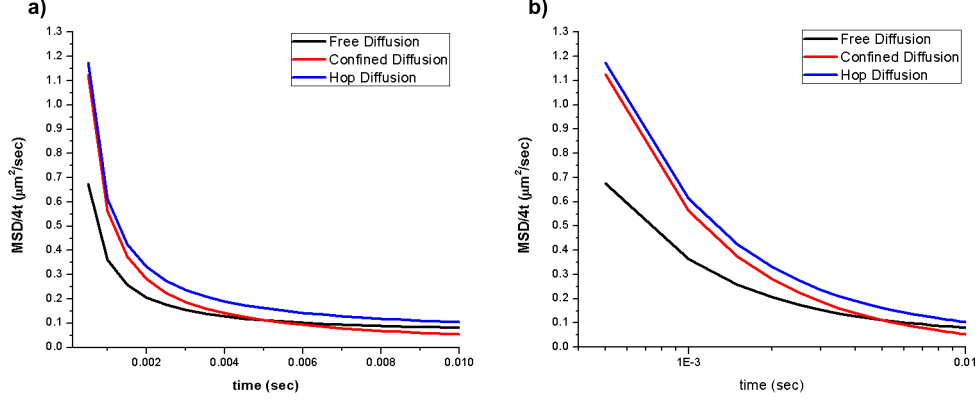


Figure 2.10: Graphical comparison of the fitting models adopted in this study, presented in $MSD/4t$ vs. t representation. The plots were obtained from the expression in Eq. 2.14, 2.15 and 2.16 using the following parameters: $\delta_{x^2+y^2} = 25$ nm, $D = 0.05 \mu m^2/s$, $D_\mu = 0.01 \mu m^2/s$, $D_M = 0.05 \mu m^2/s$, $\tau = 0.025$ s. Time in linear (a) and logarithmic (b) scale.

the fit quality. The choice of BIC, rather than the Akaike Information Criterion (AIC) [135] is due to the dependence of the penalty for the number of parameters k to the number of points n used in the model ($\ln(n)k$ for BIC vs. $2k$ for AIC). This should facilitate the selection of the most likely model for increasing number of points. Furthermore, in BIC each model starts with the same probability of being realized, whereas in AIC the prior probability scales as the number of parameters to be derived, which could penalize the hop diffusion model too much with respect to the others [134]. The Relative Likelihood of each model against the most probable one was also calculated using $\exp[(BIC(M) - BIC(M_{min}))/2]$ (where M represents any arbitrary model, whereas M_{min} corresponds to the model with the lowest BIC). A metric dubbed as “Fit Quality” is introduced. For each fitting parameter, its p-value is calculated by the code (Wolfram Mathematica), and the fit is deemed “bad” if any of the parameter verify $p > 0.34$. Essentially, this value estimates how likely it is that the true value of each parameter will be equal to the estimated value. It follows that if the confidence interval is too large, this will not be true. The value of 0.34 ($\sim \sigma$) was chosen to avoid being too restrictive.

For each track, all the diffusion models were fitted to the data. The model with the highest Relative Likelihood (or smaller BIC), provided it also respected the “Fit Quality” condition, is then chosen as the correct model for each track. It is therefore possible to build a statistic of tracks belonging to each model, to “segment” the tracks by their diffusion mode.

The most representative model for each experiment was determined by applying

the aforementioned fitting operation to the ensemble average of the tracks, that is, by averaging the values of $MSD_{app}/4t$ and $\sigma^2(t)/4$ at each timepoint, across all the tracks. In most of the cases, we found that the diffusion mode which described the majority of the tracks, also fitted well the ensemble average, expectedly.

This analysis was performed using a code written in Mathematica 11, developed in close collaboration with Dr. B. Christoffer Lagerholm.

2.3 Fluorescence Correlation Spectroscopy

2.3.1 Instrumentation

Fluorescence Correlation Spectroscopy (FCS) experiments in this work were carried out using two different setups: a RESOLFT system, especially modified to enable STED microscopy (Abberior GmbH), and a Zeiss 880 Laser Scanning Microscope (Carl Zeiss Jena GmbH).

The first setup was used mainly to carry out FCS experiments in combination with STED super-resolution microscopy, in order to obtain a range of diffusion coefficients measurements at different spot sizes. Sample fluorescence was excited through the use of a pulsed 640 nm diode laser (PicoQuant) at 80 MHz repetition rate and 80 ps-long pulses. Focal spot diameters below the resolution limit were obtained, following the STED-microscopy principle, using an emission depletion beam with a wavelength of 755 nm, produced by the output of a tunable pulsed (80 Mhz repetition rate) laser source (MaiTai HP titanium-sapphire, Spectra-Newport). The depletion beam was doughnut-shaped by a vortex phase-plate (RPC Photonics). FCS autocorrelation curves were calculated by a hardware correlator (Flex02-08D, Correlator.com) [136, 137, 138].

Confocal FCS measurements on the Zeiss 880 LSM were carried out exciting the sample using a 633 nm neon-argon laser and a 488 nm laser. The autocorrelation curves, in this case, were calculated by the ZEN Blue software (Carl Zeiss Jena GmbH), measuring the fluctuations in fluorescence signal for 10second in a variety of spots.

2.3.2 Data Analysis

Extracting information on the dynamics and the photophysics of the fluorescent molecules diffusing in the sample through FCS is possible through the calculation of the second order autocorrelation function $G(t_c)$ (henceforth simply referred to as

autocorrelation function or curve) of the detected fluorescence signal $I(t)$. The autocorrelation curves originating from the fluctuations of the fluorescence signal within the observation spot are the result of the superimposition of all the phenomena that could modify the emission. These include, but are not limited to, bleaching, triplet blinking and of course the transit of fluorescent molecules within the observation area. [16, 17, 18]. However, in relation to the experiments considered, it is possible to reduce the problem through a series of reasonable assumptions. First of all, since there is no influence of the particle photophysics on the diffusion of the particles, it is possible to separate these contributions [139, 140]:

$$G(t_c) = 1 + G_N(0) \cdot G_{diffusion}(t_c) \cdot G_{photophysics}(t_c) \quad (2.19)$$

in which t_c represent the correlation time. $G_N(0)$ is the inverse of the average number of particle transiting through the observation area throughout the observation time. If no bleaching takes place during the correlation time, then it is possible to reduce the contribution of the fluorescent probe photophysics to a single exponential decay [19, 141]

$$G_{triplet}(t_c) = 1 - T + T \cdot e^{-\frac{t_c}{\tau_T}} \quad (2.20)$$

where T is the average number of molecules at the dark triplet state and τ_T is the characteristic decay time of the molecule from the excited state to this non-emitting state. Finally, the diffusion of particles in and out the observation area, assuming a strictly two-dimensional Brownian diffusion dynamic, as it is the case for the model membranes we analyzed, is represented by the function [19, 20]:

$$G_{diffusion} = \left(1 + \frac{t_c}{\tau_{diff}}\right)^{-1} \quad (2.21)$$

in which τ_{diff} is the average transit time of the fluorescent molecule through the observation spot (“diffusion time”). This assumption holds as the samples on which we applied FCS on are two-dimensional, homogeneous model membranes.

All the autocorrelation curves obtained from the FCS and STED-FCS measurements were fitted using the FoCuS-point software [142] to the following model:

$$G(t_c) = G_N(0) \cdot \left(\frac{1}{1 + \frac{t_c}{\tau_{diff}}}\right) \cdot (1 - T + T \cdot e^{-\frac{t_c}{\tau_T}}) + \text{offset} \quad (2.22)$$

the offset term is simply a baseline introduced for fitting purposes. We did not introduce any anomaly coefficient that would account for anomalous diffusion dynamics (e.g., on the cellular membrane [143]). In fact, we measured FCS correlation curves

only on homogeneous model membranes (Supported Lipid Bilayers, described in the following), where it was assumed that the fluorescent particles were freely diffusing.

In the case of fluorescent gold nanoparticles, it was necessary to adopt a two-species diffusion model, whose equations is:

$$G(t_c) = G_N(0) \cdot \left(A \cdot \left(\frac{1}{1 + \frac{t_c}{\tau_{diff,1}}} \right) + (1 - A) \cdot \left(\frac{1}{1 + \frac{t_c}{\tau_{diff,2}}} \right) \right) \cdot (1 - T + T \cdot e^{-\frac{t_c}{\tau_T}}) + \text{offset} \quad (2.23)$$

where A is a factor (less than one) representing the fraction of the particles diffusing with average transit time $\tau_{diff,1}$, while the rest (1-A) diffuse with $\tau_{diff,2}$. In both model, the triplet relaxation time τ_T has been fixed at 5 μ s.

In order to verify that the two-component model was preferable to the single component one, we evaluated the goodness of the fit by calculating the Aikaike Information Criterion (AIC) for each model [95, 135]. The calculation of this quantity follows the formula:

$$AIC = 2k + n \ln(RSS) \quad (2.24)$$

where k is equal to the number of parameters to be estimated by each model (k=4 for the one component model, k=6 for the two component model), n is the number of points that were fitted, and RSS is the residual sum of squares between the model and the fitted data. Naturally, the lower this coefficient, the better fit to the real data is the model analyzed [135, 134]. The relative likelihood of each model was also calculated using the formula:

$$\text{Relative Likelihood} = e^{\left(\frac{AIC_{\text{minimum}} - AIC_{\text{model}}}{2} \right)} \quad (2.25)$$

In the STED-FCS measurements, once the average transit times τ_{diff} were known, it was possible to calculate the apparent diffusion coefficients. This quantity is especially useful when carrying out STED-FCS measurements, as it is independent of the observation spot size, thus providing a way of probing the diffusion behaviour of the same diffusing species at different length scales [12, 20, 144]. This calculation follows the formula [20, 143]:

$$D_{app} = \frac{d^2}{8 \cdot \ln(2) \cdot \tau_{diff}} \quad (2.26)$$

where d is the diameter of the observation spot size, defined at half the maximum of the Gaussian intensity profile of the focal spot [76, 20]. For the FCS measurements

carried out on the Zeiss 880 LSM microscope, the spot size diameter d was calculated following the Abbe formula Eq. 1.2 in the case of $NA_{condenser} = NA_{objective}$:

$$d = 0.5 * \lambda_{exc} / NA_{objective} \quad (2.27)$$

given the geometry of the system. Since the objective we used has a NA of 1.2, the resulting spot sizes are ≈ 200 nm for 488 nm excitation, and ≈ 270 nm for 633 nm excitation. In the case of STED-FCS measurements, carried out on the RESOLFT Abberior system, the observation spot diameter in confocal microscopy case was calculated from the FWHM of the confocal images of 20nm fluorescent Crimson Beads (Life Technologies), and found to be $d=240$ nm [67]. The observation spot size was reduced in a controlled way by increasing the power of the STED laser. The calibration of the observation spot size was done empirically by studying the diffusion of a fluorescent lipid probe which is assumed to diffuse freely on a homogeneous lipid bilayer. The reason for this resides in the fact that the fluorescent dyes used for FCS measurements (KK114, Abberior Star 635) could have a different depletion behaviour compared to the aforementioned fluorescent beads. Furthermore, while imaging experiments rely on pulsed depletion beams, in STED-FCS the depletion is performed in continuous wave regime. For these reasons, we chose to measure the STED spot sizes through FCS measurements, while in general fluorescent bead measurements would be preferable to calibrate the resolution of a STED microscope. We therefore produced a homogeneous Supported Lipid Bilayer (see Section 2.4) composed of DOPC, with the addition of 0.01 mol% KK114-DPPE, a fluorescent lipid analogue. The diffusion coefficient was then measured with the conventional confocal microscopy observation spot size, and at the different STED laser powers adopted in the experiments. The focal spot diameters at each STED laser power level are then easily calculated, since it has been shown that in FCS the transit times scale linearly with the square of the observation spot diameter (assuming a freely diffusing calibration probe) [24, 145, 146]:

$$d_{STED} = d_{conf} \cdot \sqrt{\left(\frac{\tau_{diff_{STED}}}{\tau_{diff_{conf}}} \right)} \quad (2.28)$$

where d_{conf} and $\tau_{diff_{conf}}$ are the spot diameter and transit time in the confocal case, while d_{STED} and $\tau_{diff_{STED}}$ are the same quantities in the STED microscopy case.

2.4 Supported Lipid Bilayers (SLBs)

2.4.1 Lipids

DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), cholesterol and the biotinylated lipid analogues DSPE-PEG(2000)-Biotin (DSPE: 1,2 - distearoyl - sn - glycerol - 3 - phosphoethanolamine, PEG: polyethyleneglycol (MW: 2kDa) and biotin) and DOPE-cap-Biotin (DOPE: 1,2 - dioleoyl - sn - glycerol - 3 - phosphoethanolamine, cap: short C6-linker, and biotin) were purchased from Avanti Polar Lipids. The fluorescent analogues Atto488-DSPE and Atto488-DPPE (DPPE: 1,2 - dipalmitoyl - sn - glycerol - 3 - phosphoethanolamine) were purchased from Atto-Tec GmbH. KK114-DPPE was obtained from a custom made stock available in the laboratory [24]. Lipids were stored as solution of different concentrations at -20 °C in nitrogen-filled glass flasks to avoid evaporation of the solvent, and briefly sonicated prior to utilization.

2.4.2 Membrane preparation

Supported Lipid Bilayers (SLBs) were prepared by spin-coating the lipid solutions on a glass substrate. This method of relatively recent invention, first mentioned in [147] has been preferred to other methods such as vesicle deposition as it allows a faster preparation time with repeatable results, as verified in several FCS-based studies [24, 148, 138].

The preparation protocol is as follows. A mixture of 1mg/ml total lipid concentration, dissolved in 1:1 chloroform:methanol solvent was prepared with the desired ratio of lipids. The glass supports (Menzel-Glaser, 1.5mm thickness, 25 or 18mm diameter) were piranha solution-cleaned using a 2:1 mixture of 96%(w/w) H_2SO_4 : 30% (w/w) H_2O_2 for 90min and extensively rinsed with ultrapure water. Depending on the glass support size, an appropriate amount of lipid solution (15 μl for the 18mm diameter, 25 μl for the 25mm diameter) was dropped on it, right before spinning at 3000 rpm. This ensured rapid evaporation of the solvent, and formation of the bilayer on the glass support, which was kept hydrated with a 18mM HEPES, 150mM NaCl, pH 7.35 buffer. SLBs prepared this way were found to be stable for several hours.

2.5 Single Particle Tracking tags

2.5.1 Fluorescent Labelling of Gold Nanoparticles

Streptavidin-coated, 40nm diameter gold nanoparticles (BBI Solutions), were purchased as a 10OD solution, corresponding to approximately 3mM, dissolved in a 2mM sodium phosphate, pH7.2 buffer. In order to make them detectable through FCS and STED-FCS, without altering the biotin-binding properties of the protein coating, we tagged them using the NHS-ester version of the Abberior STAR 635 dye and exploiting its reactivity with the amine group of the proteins. We prepared a solution of 3 μ M gold nanoparticles and 10 μ M of the NHS-ester dye in PBS buffer. To initiate the NHS ester-amine binding reaction we raised the pH of the solution with the addition of 10% w/w of 1M NaHCO₃ solution, and incubated at room temperature for 1h. In order to remove the excess dye, we placed the final product in a dialysis membrane (Thermo Scientific, 10kDa MWCO) and dialyzed the final product overnight for at least 24h in 4l of PBS buffer at 4 C.

Finally, we tested against the presence of energy transfer mechanisms between the fluorescent dye and the gold nanoparticle, that could potentially alter the fluorescent properties of the Abberior 635 dye. We verified that the fluorescence lifetimes τ of fluorescent gold nanoparticles and free dyes in solution were comparable. We performed these measurements on a Leica SP8 STED microscope, and analyzed the data using the SymPho Time software (PicoQuant GmbH). We collected data for a solution of 3 μ M solution of Abberior 635-NHS ester in the same buffer used to keep SLBs hydrated (18mM HEPES, 150mM NaCl, pH 7.35 buffer), and for several immobile fluorescent gold nanoparticles on a SLB surface. Since no significant differences were found between these samples ($\tau = 3.85 \pm 0.05$ nsec for fluorescent gold nanoparticles and $\tau = 3.76 \pm 0.02$ nsec for the dye in solution, $\lambda_{max}=655$ nm and $\tau_T=5-10\mu$ sec) we concluded that the labelling protocol was valid, not having significantly altered the properties of the fluorescent tag, and the nanoparticles were deemed appropriate to use [67].

2.5.2 Protein and Lipid Tags

Abberior STAR 635P-conjugated Streptavidin and Neutravidin were purchased from Abberior GmbH in a 1mg/ml aqueous solution. Mono-Trapflavidin, a monovalent, protein-engineered version of streptavidin, was provided courtesy of Prof. Mark Howarth and Dr. James Ross as a gift. The full name of this variant describes the main characteristics of this tag. The “mono” suffix comes from the fact that

three of the four subunits that make up the streptavidin molecule present amino-acid modifications to the biotin binding site that make the bond impossible, thus making it monovalent. The “trap” part is inserted to cite that the biotin-binding subunit is modified to allow a slower dissociation rate than wild-type streptavidin. Finally, the “flavidin” name was invented as a contraction of “fluorophore-friendly streptavidin”, since this particular version of traptavidin presents four solvent-exposed amine group, which can be conjugated to NHS-ester dyes [55, 56, 58]. In order to simplify the notation in the remainder of the work, the name “flavidin” will be adopted in the text. The protein solution was synthesised, conjugated to Abberior STAR 635P, NHS ester, and filtered to avoid the presence of excess dye in prof. Mark Howarth laboratory in the Biochemistry Department (University of Oxford). The solutions were provided in nominal μM concentrations, estimated through spectroscopic means.

The SLB samples were labelled with diluted protein tags, with variable dilution factors between 1:50 to 1:25 in the hydration buffer ompared to the starting solutions. The density of particles reached with this method was judged sparse enough to be able to track each particle singularly.

2.6 Cell samples

2.6.1 Culture

The cells employed in this study were PtK2 cells, an epithelial cell line originally derived from rat kangaroo (*Potorous tridactylis*) kidney tissue [149], which have been widely used for studies of membrane mobility [150]. These were cultured following our laboratory tested protocols, that is, growing them in Dulbecco Modified Eagle Serum (DMEM, Sigma Aldrich), supplemented with $\sim 15\%$ FBS and 1% L-glutamine [138]. In addition to this, we supplied the medium with a small quantity of Penicillin Streptomycin antibiotic to prevent the growth of contaminating bacterial colonies. Cells were initially grown in sterile single-use flasks, placed in a 37°C incubator in water-saturated atmosphere with 5% CO_2 .

Cells were grown to almost 100% confluency in sterile flasks, and resuspended with the use of a Trypsin-EDTA solution (GibCO) to seed them on 25mm glass converslips (Menzel-Glaser, 1.5mm thickness, 25mm diameter, 1.5mm thickness), which were previously sterylized by extensive dousing with industrial methylated spirit. The glass-grown cells were then prepared for experiments once they reached 50 to 70% confluency, initially loading them on sealed stainless steel chambers and kept hydrated with fresh L15, no phenol red buffer (Life Technologies).

2.6.2 Cell Membrane Labelling

The label chosen for Single Particle Tracking of lipids on the PtK2 cell surface were the same streptavidin-coated, 40nm diameter gold nanoparticles (BBI) used to label biotinylated lipids on Supported Lipid Bilayers. In fact, this specific label scatters enough to be distinguishable using the iSCAT microscope on the cell surface, despite the large amount of background scattering. In order to ensure correct labelling, the cells were loaded with DSPE-PEG-Biotin (Avanti) by dissolving a final concentration of 0.2 mg/ml in the overhead buffer and incubated at 37°C for 30 min. At the same time, the same cells were fluorescently labelled with Atto488-DOPE (Atto-tec GmbH) to allow for easy identification of the sample using the fluorescence channel of the iSCAT microscope. After this incubation step, the cells were rinsed with fresh buffer and incubated for 15 min at 37 °C with a final concentration of 0.6 μ M of streptavidin-coated gold nanoparticles. The reduced incubation time for this step was chosen to avoid the possibility of incorporation of the gold nanoparticles inside the cells.

During the experiments, the cells were kept at 37 °C using the temperature control chamber previously mentioned (Warner Instruments). Under this conditions, it was possible to observe a very sparse labelling of cells with gold nanoparticles ($\sim$ 1-2 particle per cell, with a sufficient number of cells successfully labelled), which is preferable in the case of single particle tracking studies, and an adequate amount of fluorescence for imaging.

Chapter 3

Gold Nanoparticles on Supported Lipid Bilayers

When using scattering based detection, it is common practice to rely on large scattering tags to enhance the contrast of the particles of interest against the background. However, the large size, and the crosslinking capabilities of such labels affect the diffusion mode of the tagged particles. We found it necessary to undertake complementary iSCAT-SPT and FCS/STED-FCS measurements to identify what kind of effect these characteristics have on the tagged lipid dynamics. The results presented in this Chapter aim to verify the viability of large gold nanoparticles as a label for Single Particle Tracking.

First, we describe the results obtained through iSCAT-based SPT on biotinylated phospholipids tagged with streptavidin-coated gold nanoparticles. At first, we describe 40 nm gold nanoparticle-tagged lipids tracking obtained at low sampling rates (100Hz), as complementary measurement to FCS and STED-FCS experiments. Through fluorescent tagging of the protein coating of the gold nanoparticles, the same samples could also be observed using fluorescence microscopy. Therefore, the diffusion of the gold-tagged lipids could also be monitored using FCS and STED-FCS. These results, which are the main object of [67], have also helped to gain a deeper understanding on how the crosslinking capabilities and size of a scattering tag and its size influence the results of a particle tracking experiment.

In the second part of the chapter, we present the results of SPT experiments performed on a very similar sample, but at higher sampling rates (2 kHz). By increasing the temporal resolution, however, we show how the measurement errors affect Single Particle Tracking analysis. Thus, the need for a more detailed data analysis protocol arises, as explained in the Introduction and Materials and Methods. We apply this protocol in this chapter for the first time.

3.1 Effect of gold-tagging on phospholipid diffusion

3.1.1 Gold Nanoparticle tags

In the first part of the project, we investigated the influence of large scattering tags, such as nanometer-sized gold nanoparticles, on the lipid dynamics on biological membranes. In fact, as we discussed in the Introduction chapter, the use of tags is an (almost) essential feature of every Single Particle Tracking experiment. When dealing with scattering-based detection gold nanoparticles are the most favoured choice, thanks to their unsurpassed light scattering properties [53]. However, these tags may favour crosslinking of different lipids at the same time given their large size, hindering the motion of the target molecules [63, 54, 53]. These kinds of phenomena, on the other hand, are supposedly not observed when directly tagging the lipids with small fluorescent dyes.

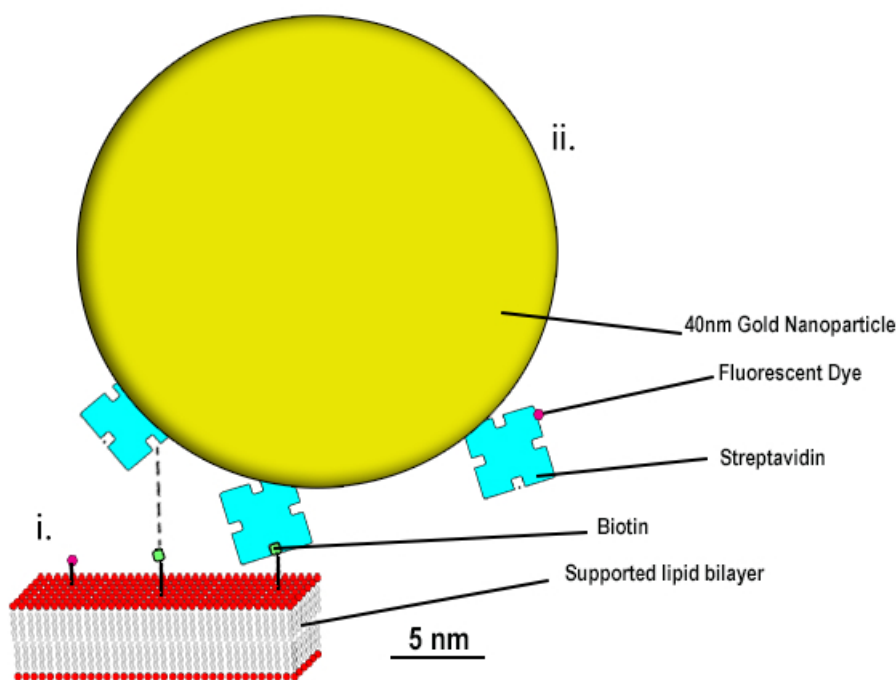


Figure 3.1: Size comparison, to scale, of the tags used in this experiment on a homogeneous lipid bilayer (red: lipid head groups, white: lipid chains). The phospholipids were either tagged with (i) a small organic dye, or (ii) with a streptavidin-coated 40 nm gold nanoparticle, whose streptavidins were stochastically tagged with the Abberior STAR 635 dye. The dashed line represent the possibility of the gold nanoparticle to crosslink several molecules.

In order to attempt a direct observation, and comparison, of the effect of these

different tagging modalities in a controlled environment, we studied the diffusion of streptavidin-coated 40 nm gold nanoparticles on a homogeneous DOPC Supported Lipid Bilayer (SLB) (lipid concentration 1 mg/ml in a 50:50 chloroform:methanol solution). The bilayer is enriched with 0.5 mol% of two different biotinylated phospholipids, DOPE-cap-Biotin and DSPE-PEG(2000)-Biotin (see Materials and Methods for the full nomenclature). We proceeded to track the particles at a slow sampling rate (100 Hz), using the iSCAT setup installed in Prof. Eggeling’s laboratory. The same particles were also tagged with a fluorescent dye (Abberior STAR 635), in order to study their dynamics using Fluorescence Correlation Spectroscopy (FCS), also in combination with super-resolved Stimulated Emission Depletion microscopy (STED-FCS). The comparison of these tags is shown in 3.1. This made it possible to compare their motion with a single fluorescent phospholipid analogue (DPPE-KK114), using fluorescent-based detection.

3.1.2 Confocal FCS experiments

We first present the results of the confocal FCS measurements for the fluorescent lipid analogues and the gold nanoparticles. As it was highlighted from Fig. 3.2, a significant difference in average transit time between the two different tags can be discerned. This is manifested in a shift towards longer correlation times. It is therefore evident that the use of the large, potentially crosslinking, gold nanoparticle tags effectively reduces the mobility of the target lipids.

It was necessary to adopt different models to fit the data originating from the fluorescent lipid analogues, and the fluorescent gold-tagged biotinylated lipids. We adopted a model with one diffusing component (Eq. 2.22), and a model with two diffusing components (Eq. 2.23) to explain these different behaviours. The fit parameters of the most appropriate model are summarized in Table 3.1. The goodness of the fit was evaluated by calculating the AIC of each model, and from this deriving the Relative Likelihood values [135]. From this analysis, we derive that the KK114-DPPE FCS data are more likely described by a single diffusing component model, with an average transit time $\tau_{diff} = 2.0 \pm 0.01$ ms (sample size=10). With the same procedure, it was verified that both the gold-tagged phospholipid species autocorrelation functions are better described by the two-diffusing component model. In particular, fluorescent gold nanoparticle-tagged DSPE-PEG-Biotin shows $\tau_{diff,1} = 2.41 \pm 0.2$ ms and $\tau_{diff,2} = 120 \pm 30$ ms (sample size=10), and for DOPE-cap-Biotin we obtain $\tau_{diff,1} = 1.7 \pm 0.2$ ms and $\tau_{diff,2} = 132 \pm 30$ ms (sample size = 10). In both gold-tagged phospholipids, the Relative Likelihood of the one-component model is negligible.

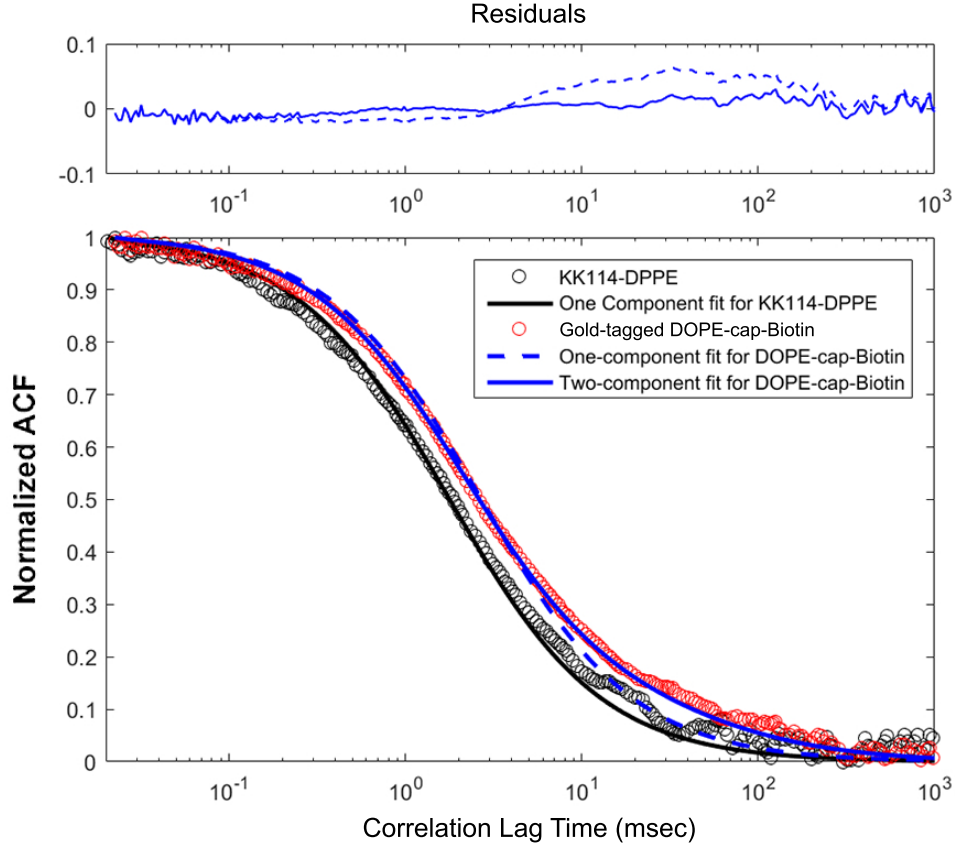


Figure 3.2: Comparison of representative, normalized confocal FCS data and fitting models for a gold-nanoparticle tagged phospholipid and a fluorescent lipid analogue. The autocorrelation function for a gold-tagged DOPE-cap-Biotin lipid (red circles) is better described by a two-component model, rather than one component (residuals of the fit shown in top panel). On the other hand, the autocorrelation curve for the fluorescent lipid analogue KK114-DPPE is well represented by a single component fit.

The nature of the “fast” component, whose average transit times is comparable with those of fluorescent dye-tagged lipids, is quite puzzling. The most likely hypothesis for this would be the presence of free fluorescent streptavidin in solution, which could directly tag the biotinylated lipids. Another possibility is the presence of unspecifically membrane-bound fluorescent gold nanoparticles which diffuse much faster than specifically bound ones. The latter was ruled out by further experiments, which show that no correlating signal could be observed from a sample without biotinylated lipids, after the addition of a solution of fluorescent gold.

We confirmed the presence of free streptavidin in the gold nanoparticle solution by performing Native-PAGE Electrophoresis on the gold solution as supplied by the vendor, and on a pure streptavidin sample. This experiment was performed by loading

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Table 3.1: Summary of the FCS Autocorrelation Function fit parameters mentioned in the text for KK114-DPPE, fluorescent gold-tagged DSPE-PEG(2000)-Biotin and DOPE-cap-Biotin. The value of A_1 is the amplitude of the component corresponding to the “fast” component in the model with two diffusing components. No value for A_2 is reported, since $A_1 + A_2 = 1$. The “excess biotin” lines refer to the fact that the gold nanoparticles are streptavidin-coated, and therefore saturation of the binding sites with excess biotin in solution was attempted.

Sample	A_1	$\tau_{diff,1}$ (ms)	$\tau_{diff,2}$ (ms)
KK114-DPPE	1	1.9 ± 0.2	-
Fluorescent gold-tagged DSPE-PEG(2k)-Biotin	0.87 ± 0.04	2.4 ± 0.2	110 ± 30
Fluorescent gold-tagged DSPE-PEG(2k)-Biotin (plus excess biotin)	0.8 ± 0.05	1.9 ± 0.1	130 ± 40
Fluorescent gold-tagged DOPE-cap-Biotin	0.87 ± 0.03	1.7 ± 0.2	130 ± 30
Fluorescent gold-tagged DOPE-cap-Biotin (plus excess biotin)	0.84 ± 0.06	2 ± 0.2	130 ± 40

between 1 μg and 10 μg of the samples in the wells of a polyacrylamide gel (NuPage 4-12%Bis-Tris Gel, Life Technologies), and were marked with NuPage LDS sample buffer diluted 20 times. A protein reference sample (Bio-Rad Precision Plus Protein Standard) was also loaded, to provide a calibration for the molecular weight. The gel was run (100V for 60min), stained (Simply Blue SafeStain, Invitrogen) and scanned with a UV-lamp to reveal the composition of the samples (Fig. 3.3). Due to their large size, and probably electrostatic aggregation due to the experimental procedure, the gold nanoparticles do not manage to diffuse into the gel, forming a band at the bottom of the well in lane 2 of Fig. 3.3. However, we notice that both the gold nanoparticle sample in lane 2, and the Alexa 488-conjugated streptavidin in lane 3 share similar bands, with the largest component in correspondence of the 50 kDa band of the reference scale, roughly the molecular weight of streptavidin (≈ 55 kDa).

It is reasonable to expect that the free streptavidin hereby detected could be tagged by the same protocol as the gold nanoparticle fluorescent tagging. In fact, we recall that the target of this procedure are the protein coating the nanoparticle itself. We deduce from these experiments that in order to eliminate the fast component from the FCS curves, more sophisticated purification procedures are required. We did not attempt such procedures, since this only affects the fluorescence-based experiments,

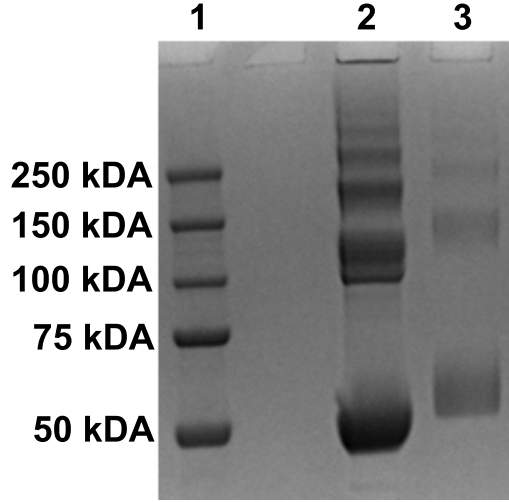


Figure 3.3: Results of gel electrophoresis of the following samples: (1) reference sample (Bio-Rad Precision Plus Protein Standard), (2) 3mM 40 nm streptavidin-coated gold nanoparticle sample, as supplied by the producer, and (3) 20 μ M Alexa 488-tagged streptavidin. On the side, the weight scale as indicated by the reference sample producer is reported.

since the scattering from the gold nanoparticles overwhelms the scattering from the smaller streptavidin.

From these controls, we can assume that the slow component must originate from the diffusing gold-tagged phospholipids. We notice that such component is consistently smaller than the fast component ($\sim 10\%$ of the total curve amplitude), given the sparsity of the gold nanoparticles necessary for Single Particle Tracking. Analysis of the FCS curves through the appropriate model reveals that while KK114-DPPE, the fluorescent lipid analogue, diffuses with an apparent diffusion coefficient $D_{app} = 5.3 \pm 0.2 \mu m^2/s$, gold tagged DOPE-cap-Biotin and DSPE-PEG-Biotin data reveal, respectively, $D_{app} = 0.12 \pm 0.04 \mu m^2/s$ and $D_{app} = 0.08 \pm 0.02 \mu m^2/s$. This observed 40-to-60 fold slowing in diffusion could either be due to the size of the nanoparticles, but also the crosslinking of several biotinylated lipids by the extended gold nanoparticle [117, 63]. In an effort to reduce the effects of crosslinking, we tried adding excess free biotin in solution to saturate the binding sites on the nanoparticle surfaces. We measured the FCS autocorrelation curves for these samples as well, and analyzed using the model with two diffusing components. The fit parameters are reported in Table 3.1, for ease of comparison to the previously obtained results. FCS data showed little to no change in the average decay time of the slow component of the FCS curves ($\tau_{diff,2} = 130 \pm 40$ ms with $D_{app} = 0.08 \pm 0.02 \mu m^2/s$

for DSPE-PEG-Biotin, and $\tau_{diff,2} = 130 \pm 40$ ms with $D_{app} = 0.08 \pm 0.02 \mu m^2/s$ for DOPE-Cap-Biotin). While this control condition cannot fully exclude the persistence of crosslinking artifacts, it may still indicate that the greater size of the tag alone can induce a slower observed lipid diffusion [53].

3.1.3 STED-FCS experiments

The diffusion of phospholipids on a homogeneous membrane should be, in principle, an example of free diffusion, given the absence of anomaly-inducing obstacles. In order to ascertain whether the diffusion mode of the gold tagged phospholipids remained unaltered despite the tag, we studied the behaviour of the apparent diffusion coefficient D_{app} against the FCS observation spot diameter. We tuned the observation spot diameter using STED super-resolution microscopy, by increasing the depletion laser power. In the case of freely diffusing molecules, such as in this study, the apparent diffusion coefficient should be independent of the size of the focal spot [24].

The KK114-DPPE enriched sample was used as calibration, to determine the variation of the spot size, following Eq. 2.28. The results of the measurements on KK114-DPPE, and on fluorescent gold-tagged DOPE-cap-Biotin and DSPE-PEG-Biotin are reported in Fig. 3.4.

When recording the autocorrelation functions for the fluorescent gold-tagged samples, we notice that the whole curve shifts towards shorter correlation times (Fig. 3.4a), both in the “slow” and the “fast” components, as expected when the observation spot size is reduced. The behaviour of D for all samples is shown in Fig. 3.4b. The diffusion of the gold-tagged phospholipids could not be observed for many values of observation spot size. In fact, it would not make sense to detect the movement of 40 nm gold nanoparticles with an observation spot smaller than the diameter. However, it is important to notice that the apparent diffusion coefficient for the slow component of the gold-tagged DOPE-cap-Biotin and DSPE-PEG-Biotin autocorrelation curves is roughly independent of the observation spot size, meaning that the free diffusion behaviour of these particles is unaltered by the extended gold tag attached to it. Therefore, from the FCS experiments performed, it is possible to deduce that the only effect of the gold nanoparticles is slowing down the diffusion of the target molecule. While for KK114-DPPE the average diffusion coefficient across the different spot sizes is $D_{app} = 5.3 \pm 0.2 \mu m^2/s^2$. The same quantity calculated from the average transit times of the slow component of the autocorrelation functions for fluorescent gold tagged DOPE-cap-Biotin is $D_{app} = 0.08 \pm 0.2 \mu m^2/s^2$ and for DSPE-PEG-Biotin is $D_{app} = 0.12 \pm 0.2 \mu m^2/s^2$.

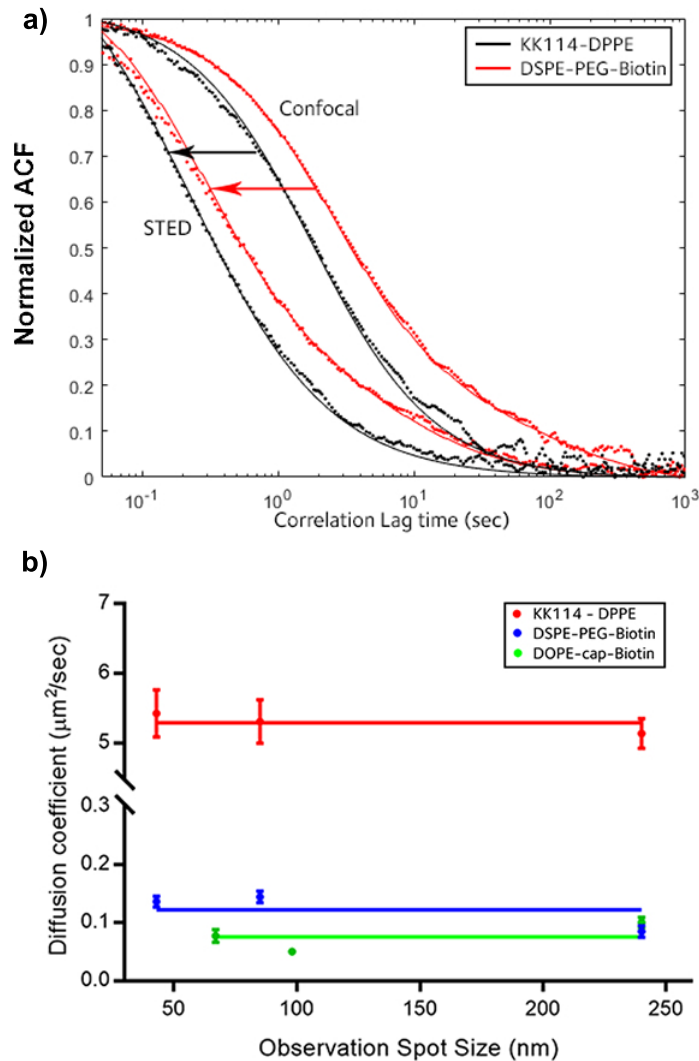


Figure 3.4: STED-FCS measurements on KK114-DPPE and gold-tagged phospholipids on an homogeneous DOPC lipid bilayer. a) Effect of spot size reduction on the autocorrelation curves for the fluorescent lipid analogue KK114-DPPE, and on fluorescent gold-tagged DSPE-PEG-Biotin; b) Plot of apparent diffusion coefficient D against observation spot size (d) for all the samples (dots with error bars). The solid lines represent the average for D across the detected points. The values of the diffusion coefficient for the biotinylated lipids refer only to the slow component of the autocorrelation functions. The DOPE-cap-Biotin sample has been recorded at different spot sizes due to different result for the calibration sample when the data were recorded.

As a further control measurement, we checked whether the binding of the Abberior STAR 635 dye to the streptavidin-coated gold nanoparticles would affect the emission lifetime of the fluorescent dye Fig. 3.5. It is in fact, extremely important that the photophysics of the dye is unaltered by the tagging operations, to obtain similar resolution improvements, that is, similar observation spot sizes [23]. The fluorescence

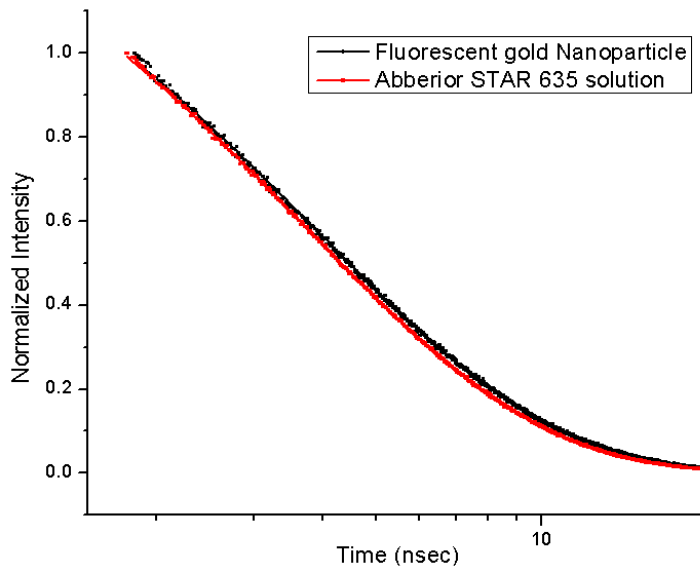


Figure 3.5: Fluorescence lifetime measurements for free Abberior STAR 635 dye in solution (red) and conjugated to streptavidin-coated 40 nm gold nanoparticles (black), measured at $\lambda = 633$ nm on a Leica STED SP8 microscope. The curve for the fluorescent dye-conjugated gold nanoparticles was taken in a Region of Interest restricted to a single nanoparticle immobilized on an SLB surface

emission lifetimes at 633 nm excitation of a solution of Abberior STAR 635, and of single fluorescent dye-tagged gold nanoparticles dropped on a SLB were measured using a Leica SP8 STED microscope. The gold nanoparticles were first visualized through confocal microscopy, and the fluorescence decay measured in a region of interest containing a single nanoparticle. The fluorescence emission of both had a peak at 655 nm. The decay times, obtained through single exponential fits, was 3.76 ± 0.02 ns for the free dye, and 3.85 ± 0.05 ns for the fluorescent gold nanoparticles, where the errors provided are the fit uncertainty. This difference in the measurements was not significant.

3.1.4 iSCAT Single Particle Tracking

The samples where the biotinylated phospholipids were tagged with the fluorescent gold nanoparticles were also observed using iSCAT microscopy. As can be seen in the insets in Fig. 3.6, the gold nanoparticles appear as dark spots over a white background, due to the nature of image formation in iSCAT. No such features were detected in the KK114-DPPE-labelled SLB, since none of the lipids have a large enough cross-section. The static features in the sample were eliminated using successive median and temporal average filtering operations (see Materials and Methods).

The particle tracks (of which we have examples in Fig. 3.6), were extracted from the 3-to-5s movies collected at 100Hz sampling frequency (0.01 s time resolution), using the μ Track algorithm included in the Matlab code TrackNTrace [70, 72]. The localization precision for these experiment was estimated at 2.6 nm by measuring the FWHM of the distribution of relative distances of two immobilized gold nanoparticles on a glass surface (Fig. 3.8) [46, 97].

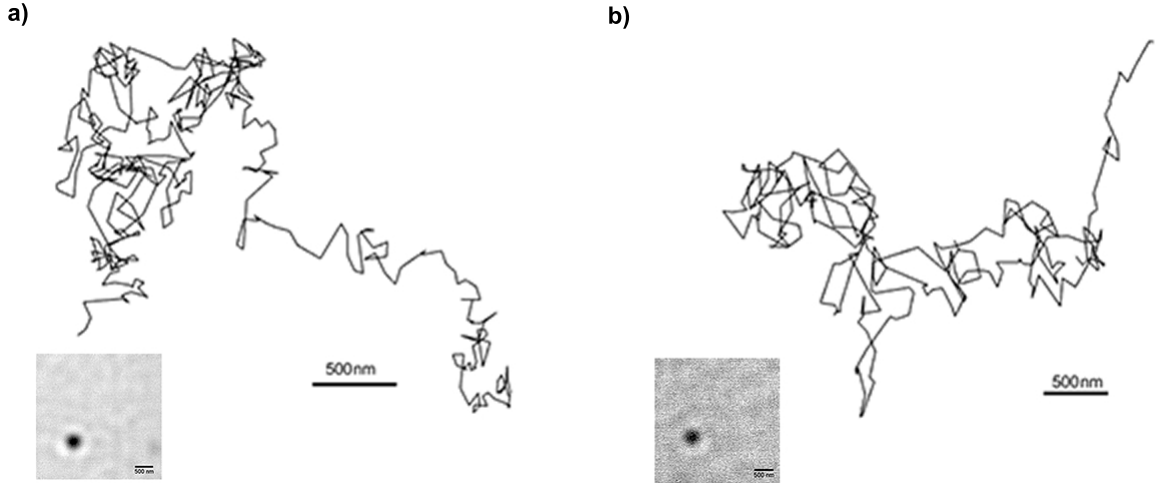


Figure 3.6: Example of particle tracks and single movie frames for a) DOPE-cap-Biotin and b) DSPE-PEG-Biotin. The movies were recorded at 100 Hz sampling frequency, and the sample was illuminated with 2.5 mW ($60 \mu W/\mu m^2$) in order to ensure near-saturation conditions, with an exposure time of 8 ms.

We collected and analyzed the tracks that contained at least 50 positions, which amounted to 97 for gold-tagged DOPE-cap-Biotin, and 63 for gold-tagged DSPE-PEG-Biotin. We calculated the Mean Squared Displacement for each of these tracks, and then ensemble-averaged them. We did not pursue a track-by-track analysis approach under the assumption that all the particles detected diffused freely on the homogeneous bilayer. We fitted the free diffusion mode (see also Eq.2.4) [151] to the first 10 timepoints of the ensemble average:

$$MSD_{app}(t) = 4D_{app}(t)t + 2\delta_{x^2+y^2}^2 \quad (3.1)$$

where D_{app} is the apparent diffusion coefficient, and $\delta_{x^2+y^2}^2$ is the localization error associated with the measurements. It is necessary to remark that we chose to fit only the first 10 points of the curve to follow the general guideline for MSD analysis of not employing more than a quarter of the data for this operation [88]. In fact, since

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The data fits well to the model in Eq. 3.1 for both samples. In both cases, the intercept (i.e., the localization error $\delta_{x^2+y^2}^2$), is essentially zero. This is an expectable outcome, since it has been extensively reported from many literature examples how iSCAT, in principle, manifests very high localization accuracy [34, 109, 110, 104, 105]. A more detailed consideration on these errors will be given in the following section. The diffusion coefficients obtained through the fits for both gold-tagged lipid species are $D_{app} = 0.17 \pm 0.005 \mu\text{m}^2/\text{s}$ for DOPE-cap-Biotin and $D_{app} = 0.31 \pm 0.01 \mu\text{m}^2/\text{s}$ for DSPE-PEG-Biotin. These results are quite different to those obtained from the FCS and STED-FCS analysis ($D_{app} = 0.08 \pm 0.02 \mu\text{m}^2/\text{s}$ for DOPE-cap-Biotin and $D_{app} = 0.12 \pm 0.04 \mu\text{m}^2/\text{s}$ for DSPE-PEG-Biotin). The origin of this disagreement could be attributed to the difference in the experimental methods employed, as described in [152]. Nevertheless, these values are still one order of magnitude lower than the diffusion coefficient for the fluorescent phospholipid analogue KK114-DPPE, as determined by FCS, confirming the effect of the heavy, crosslinking tag on the diffusion of the labelled lipids.

The unrestricted, free nature of the diffusion dynamics of the gold-tagged phospholipids is further demonstrated by the plots of $\text{MSD}/4t$ vs. t in Fig.3.7c and Fig.3.7d. In fact, given that the localization error is negligible when compared to the displacement $D_{app} * \delta t$, where δt indicates the time between two particle observations, the ratio $\text{MSD}_{app}(t)/4t$ is almost constant throughout the time interval considered [91, 95]. The graphs are represented in a double logarithmic scale to emphasize the first few points, which tend towards smaller value compared to the last ones. It is well known this is due to the camera blur effect, since the tracked particle moves during the camera integration time [92]. However, since the gold nanoparticles appear to be particularly slow, this effect is not heavily pronounced and can be neglected in the final analysis.

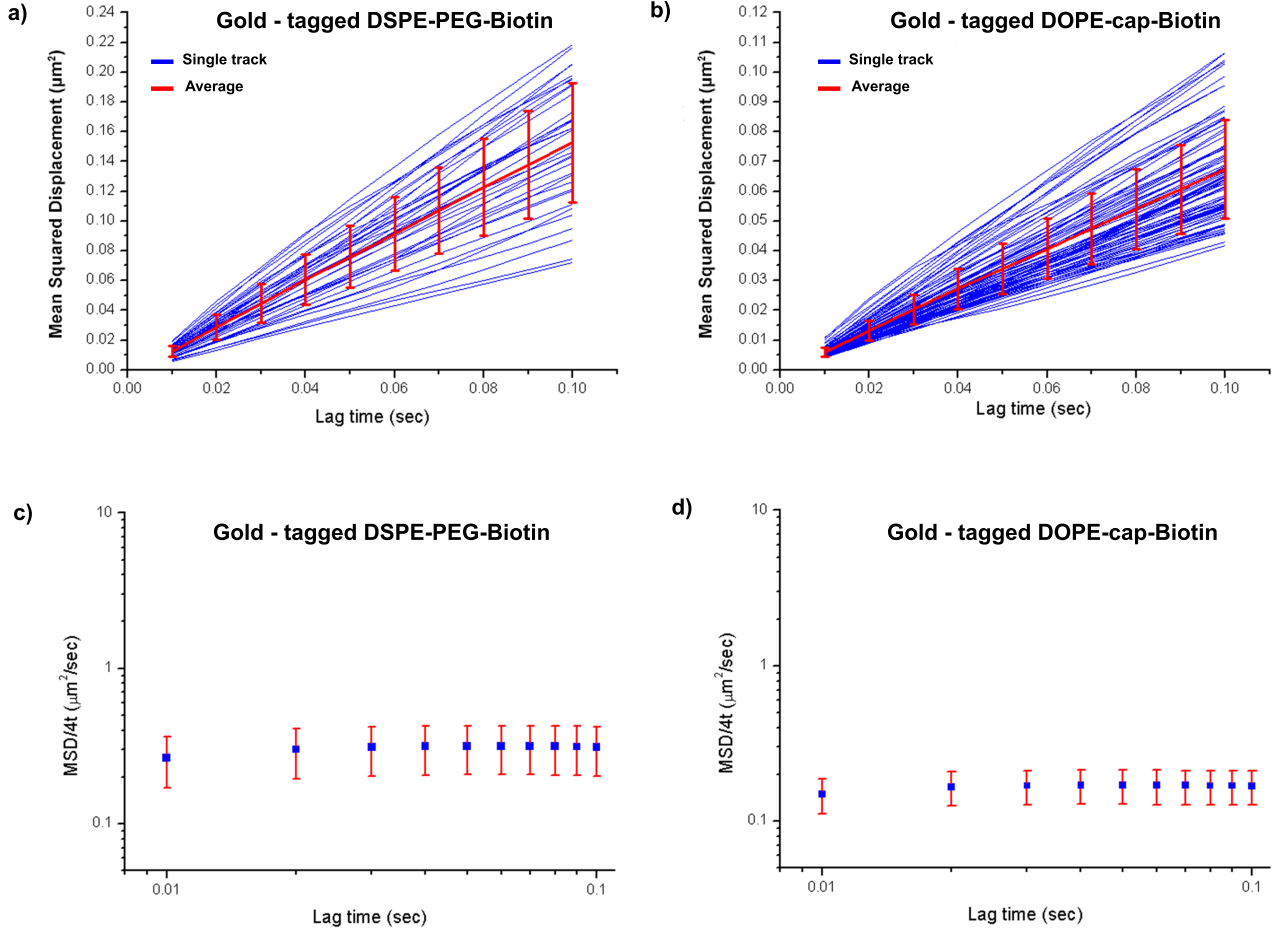


Figure 3.7: MSD vs. t and $\text{MSD}/4t$ vs. t plots for the single particle tracks of DOPE-cap-Biotin (b. and d.) and (a. and c.) DSPE-PEG-Biotin. In the MSD vs. t plots (a. and b.), the blue lines are the MSD plots of the single tracks, used to obtain the ensemble average, in red. In the $\text{MSD}/4t$ vs t plots, only the ensemble average's points, each with its relative standard deviation, are reported.

3.2 Fast sampling of gold nanoparticle diffusion

The main advantage of scattering-based detection, and of iSCAT microscopy in particular, is the possibility of reaching fast sampling rates while maintaining a good Signal-to-Noise ratio for the detected image. This is particularly true when used to track large scattering tags, such as gold nanoparticles. In the remainder of this chapter, we will show the results of high-sampling rate tracking of the same gold nanoparticles thus far observed.

The data analysis, however, has been adapted to the new experimental conditions. Given that scattering-based detection does not suffer from photophysical phenomena such as bleaching or blinking, we restricted ourselves to use only long (>500 positions),

gapless tracks. While this choice limits the number of detected tracks ultimately used for analysis, it simultaneously increases the statistical precision of the first time points of the MSD curves. This is also the first case, in this work, where we applied the advanced track analysis procedure presented in section 2.2.3.

3.2.1 Considerations on localization precision estimation

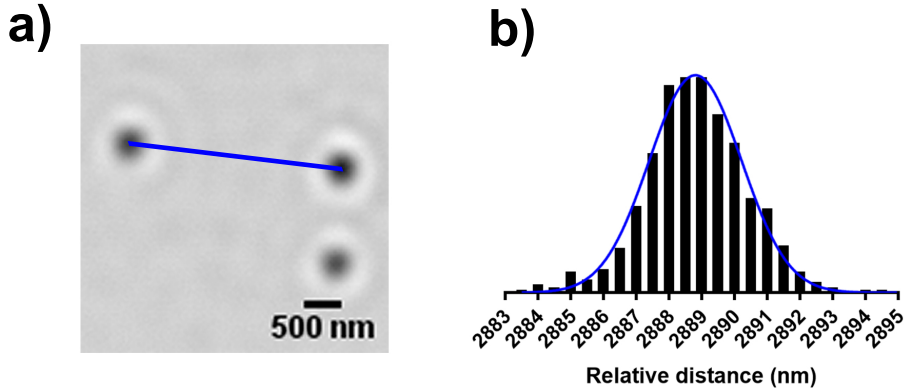


Figure 3.8: Measurement of the localization precision for immobilized 40 nm gold nanoparticles at 100 Hz framerate, as illustrated in [46, 97]. The 40 nm, streptavidin-coated gold nanoparticles were immobilized on a cover glass surface coated with a BSA and BSA-biotin film were illuminated in near-saturation conditions and imaged at 100Hz for 6 seconds (a). The relative distance between two nanoparticles is measured, and the localization precision is estimated with the FWHM of the Gaussian distribution (blue) fitted to the histogram of this value (b).

In the previous section, we have mentioned how the localization precision for the iSCAT SPT experiments has been estimated using the relative distance of two immobilized gold nanoparticles, as prescribed in [46, 97]. The localization precision was calculated as the FWHM of the Gaussian distribution fitted to the histogram of the detected relative distances, as 2.6 nm (Fig. 3.8). However, this method can sometimes underestimate the localization error. In fact, it is obvious that measuring the relative distance of two objects which are solidal to the same vibrating system, cancels out these movements.

The setup in use for these experiments was found to be affected by some instability. This becomes apparent when analyzing the “trajectory” of a single, immobile gold nanoparticle using the advanced analysis method introduced in Chapter 2 (Section 2.2.3). Briefly, the apparent Mean Squared Displacement MSD_{app} is calculated for a number of time lags, and each value divided for its corresponding time lag t , to obtain a value of $D_{app}(t) = MSD_{app}(t)/4t$. At the same time, in what we call

Step Distribution Analysis, the distributions of Squared Displacements at the same time points are fitted to a Rayleigh probability density function (Eq. 2.8), where $\sigma^2(t)/4 = D_{app}(t)$. Correction for camera blur was applied, since the presence of vibrations in the setup could affect the measurement. Finally, three different models are tentatively fitted to the data (Eq. 2.14, 2.15 and 2.16). Which of these model is the most likely to describe the motion of the particles is then assessed using the Bayesian Information Criterion, and the Fit Quality metric defined in Section 2.2.3.

The presence of spurious vibrations in the setup is noticeable from the peak at short timepoints for all the curves, as noticeable from Fig. 3.9a. We made an attempt to fit the diffusion models with the data obtained from the Step Distribution Analysis and $MSD_{app}(t)/4t$ for the immobilized gold nanoparticle positions (Section 2.2.3). Given our conditions for a “good” fit ($p < 0.32$ for all the fit parameters + error), we obtained that the best model (according to the Relative Likelihood) in the case of Step Distribution, was a free diffusion model (Fig. 3.9b) with $D_{app} = 0$ (expected for an immobile object), and localization error $\delta^2 = 7.4 \pm 0.9 \text{ nm}$. On the other hand, the most likely model for the $MSD_{app}(t)/4t$ is a confined diffusion (Fig. 3.9b), with $D_\mu = 0.02 \pm 0.01 \text{ } \mu\text{m}^2/\text{s}$, corresponding to a confinement length of $L = 34 \pm 19 \text{ nm}$ (which can be interpreted as an estimate of the amplitude of the detected movements in the setup), and a localization error of $\delta^2 = 5 \pm 2 \text{ nm}$. However, it is apparent that none of the functions can adequately describe the variation of the position of the particle over time, therefore making these difficult to accept as an adequate calibration.

The use of immobilized particles for the calibration of localization precision of SPT is quite widespread, probably because of its simplicity ([90, 85, 41, 34]). Nevertheless, as we previously pointed out, such methods should be used with caution. In fact, this kind of purposely-devised situation is very different from tracking experiments with moving particles, when phenomena like camera blurring, and the variability of Signal-to-Noise ratio affect the particle localization. While this method is valid when dealing with strong scattering, slow diffusing particles at low sampling rates, which produce a higher Signal-to-Noise ratio, we will report the localization noise as derived from model fitting for high sampling rate experiments.

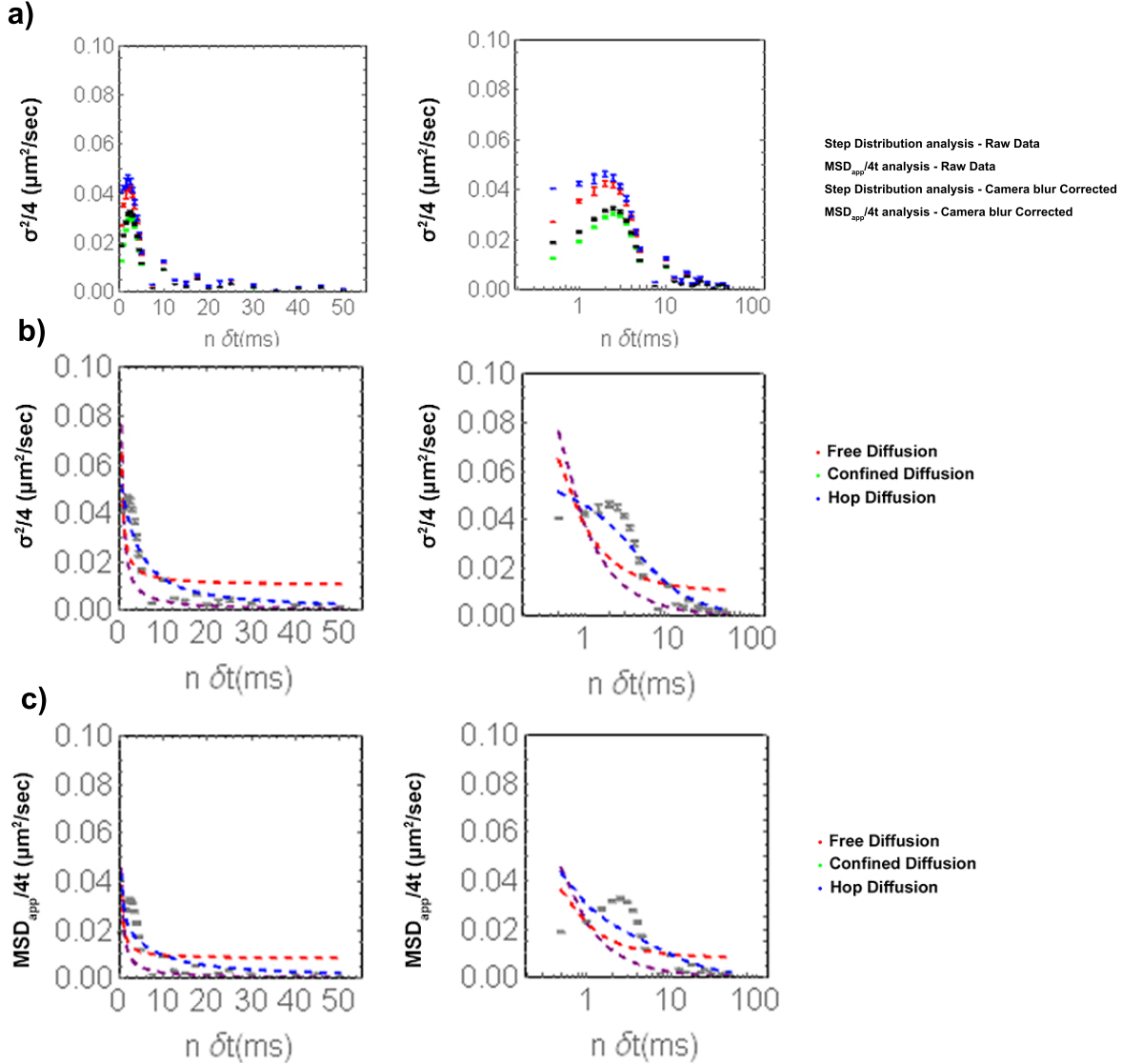


Figure 3.9: Results of MSD and Step Distribution Analysis for the detected "track" for an immobilized gold nanoparticle. (a) Comparison of $MSD/4t$ and $\sigma^2(t)/4$ curves, in "raw" and blur corrected versions. (b) Fit of the Step Distribution ($\sigma^2(t)/4$), raw and blur-corrected data with the three models used in this work: free diffusion (red), confined diffusion (green) and "hop" (mixed) diffusion. An "immobile particle" model (purple) is also fitted ($D_{app} = 0$). (c) Fit of the $MSD_{app}(t)/4t$, raw and blur-corrected, using the same models.

3.2.2 Tracking and data analysis for gold nanoparticle-tagged lipids on SLB

The 40 nm diameter streptavidin-coated gold nanoparticles were used to label the biotinylated, saturated phospholipid DSPE-PEG(2000)-Biotin on an homogeneous SLB. The lipid composition of the bilayer was 74.5 mol% DOPC, 25 mol% Cholesterol, 0.5 mol% of either of the biotinylated lipids, total lipid concentration 1 mg/ml in a

50:50 methanol:chloroform solution. The sample was illuminated using the 650 nm laser on the iSCAT setup, scanned over a $6.36 \times 6.36 \mu\text{m}^2$ area. Movies of the diffusing particles were recorded at 2 kHz for 2 s (4000 frames), and the particles were tracked using the Imaris software (Bitplane AG), selecting tracks which were longer than 500 positions. A total of 55 tracks were detected, and are reported in Fig. 3.10.

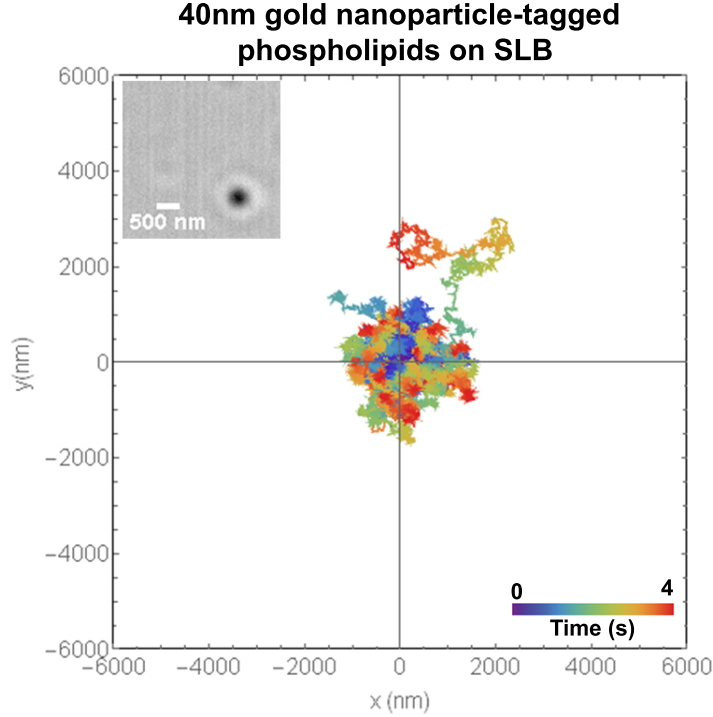


Figure 3.10: Detected tracks for 40 nm Gold Nanoparticles diffusing on a SLB, recorded at 2 kHz sampling rate. The tracks are remapped to start at (0;0) coordinate, and go from blue ($t=0$) to red ($t=4$ s). In the inset, the image of a 40 nm gold nanoparticle on the SLB, visualized at the imaging conditions exposed in the text.

The particle tracks were analyzed following the procedure presented in Section 2.2.3, to obtain values of $MSD_{app}(t)/4t$ and $\sigma^2(t)/4$ as derived from the Step Distribution analysis. An exemplary track is plotted in Fig. 3.11a. Data extracted from the $MSD_{app}(t)/4t$ and Step Distribution Analysis are plotted in Fig. 3.11b, with and without the correction for camera blur.

In Fig. 3.11a, it is possible to see the effect of the camera blur correction on the data to be analyzed. As we anticipated in Section 1.2.5, correcting for the negative contribution of camera blur, introduces a positive offset to the data (see Eq. 2.5). However, this effect is much more pronounced on short timescales, which are also the most affected by localization noise. Camera blur-corrected data were used for further analysis, and the the models described in 2.2.3 were fitted to them. The values for

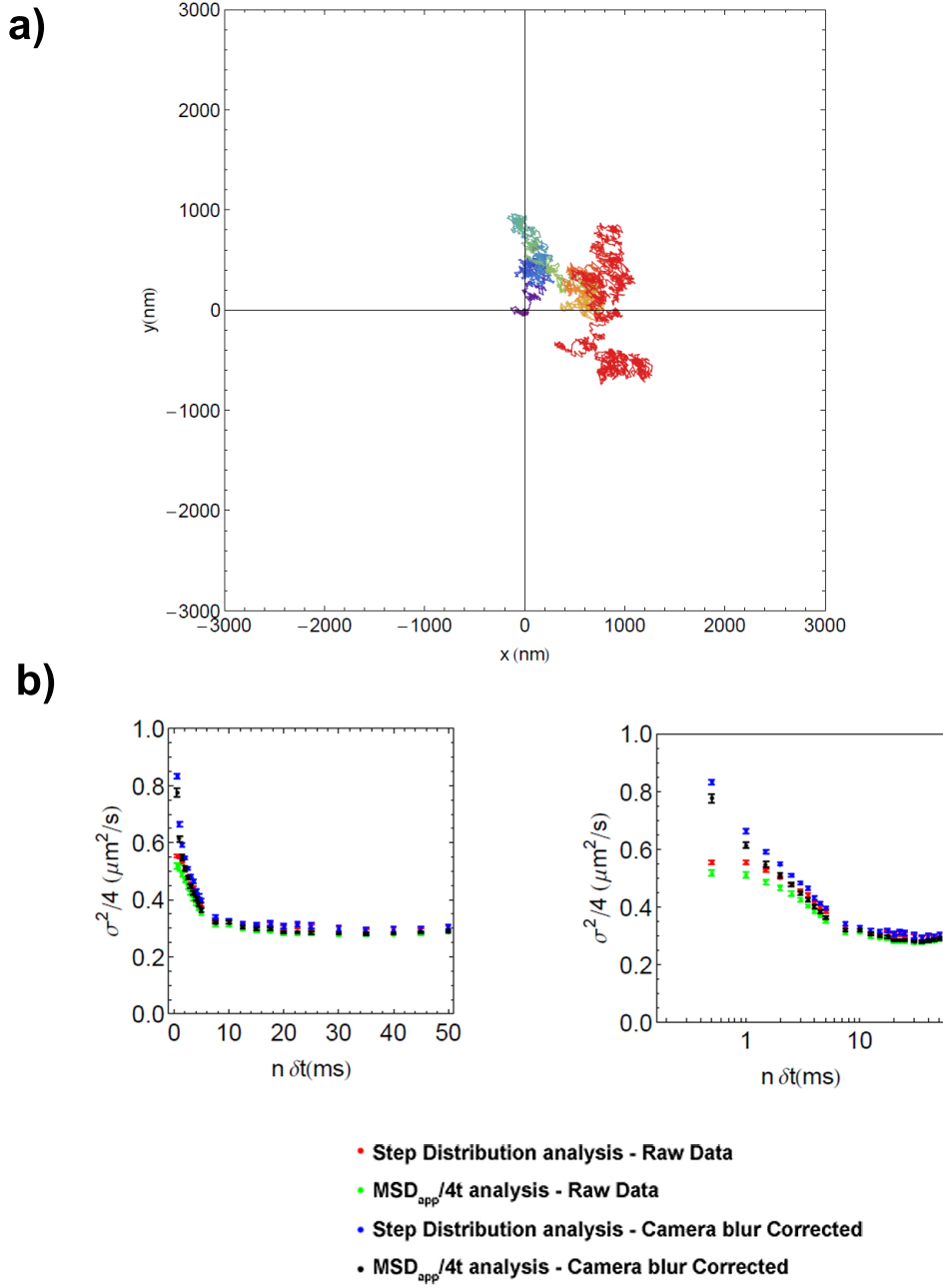


Figure 3.11: Example track of a 40 nm gold nanoparticle diffusing on an homogeneous SLB, and extracted data to be analyzed. a) Trajectory of a 40 nm streptavidin-coated gold nanoparticle diffusing on a DSPE-PEG-Biotin enriched SLB, tracked for 2 seconds at 2 kHz sampling rate. The track is color-coded to go from blue ($t=0s$) to red ($t=2 s$). The track is composed of 4000 positions b) Linear (left) and Log (right) time-axis plots of the $\sigma^2(t)/4$ (red) and $MSD_{app}(t)/4t$ (green), extracted from the track. In blue and black, the same data, after the application of the camera blur correction, for $\sigma^2(t)/4$ (red) and $MSD_{app}(t)/4t$ respectively.

the fit parameters are reported in Table 3.2 for the Step Distribution Analysis, and Table 3.3 for the $MSD_{app}(t)/4t$ data.

The fits in Fig. 3.12 and 3.13 reveal that the hop diffusion model looks like the best model to describe the particle motion. In order to give a statistical grounding to this statement, we verified the goodness of the fits by calculating the Relative Likelihood of each model. A summary of the parameters and the results of the statistical tests is given in Table 3.2 and 3.3. The fact that a mixed diffusion model would be the most likely, although in contrast with previous results, should not be surprising. Indeed, the transient ($\tau = 1.2$ ms) nature of the confinements lead us to believe that they could not be observable at slow framerates, like the ones employed before. These phenomena are most probably due to the presence of defects on the surface of the lipid bilayer.

From these results, we can already see how the simple “goodness of fit” estimation is not sufficient to evaluate the appropriateness of the chosen model for analysis. Furthermore, the localization errors, calculated through all the models, consistently appear to be larger than the value obtained from the immobilized gold nanoparticles. This can be expected, given that the Signal-to-Noise ratio for moving particles should be lower than the same particle when immobilized. Nevertheless, it still appears to be well below the diffraction limit of light, as it is expected of iSCAT microscopy [34].

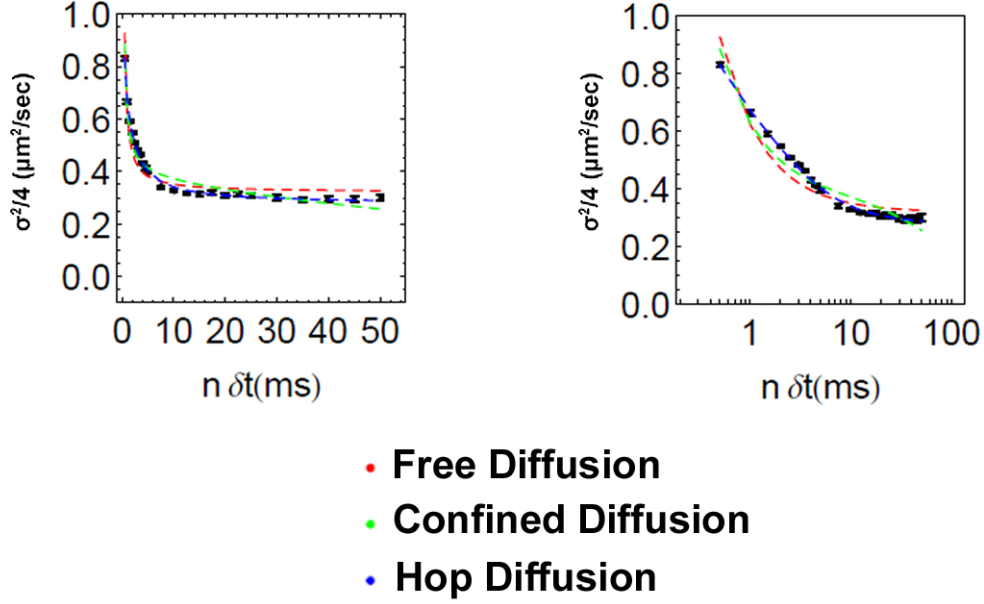


Figure 3.12: Results of the fit of the blur-corrected $\sigma^2(t)/4$ values extracted from the Step Distribution Analysis for the example track in Fig. 3.11. The dashed lines are the fits to the data of the different diffusion models adopted in this work.

Table 3.2: Results for the fitting of the Step Distribution $\sigma^2(t)/4$ curves for the example track (Fig.3.11a) with the different diffusion models

Fit Model		Free: $D + \frac{\delta_{x^2+y^2}^2}{2nt_{lag}}$	
Relative Likelihood	Fit Quality	D ($\mu m^2/s$)	δ (nm)
$2 * 10^{-16}$	Good	0.32 ± 0.01	25 ± 1

Fit Model		Confined: $D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta_{x^2+y^2}^2}{2nt_{lag}}$			
Relative Likelihood	Fit Quality	D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
$9 * 10^{-14}$	Good	0.38 ± 0.02	57.3 ± 0.02	510 ± 70	23 ± 1

Fit Model		“Hop” diffusion: $D_M + D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta_{x^2+y^2}^2}{2nt_{lag}}$				
Relative Likelihood	Fit Quality	D_M ($\mu m^2/s$)	D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
1	Good	0.28 ± 0.01	0.43 ± 0.06	1.3 ± 0.2	81 ± 8	14 ± 2

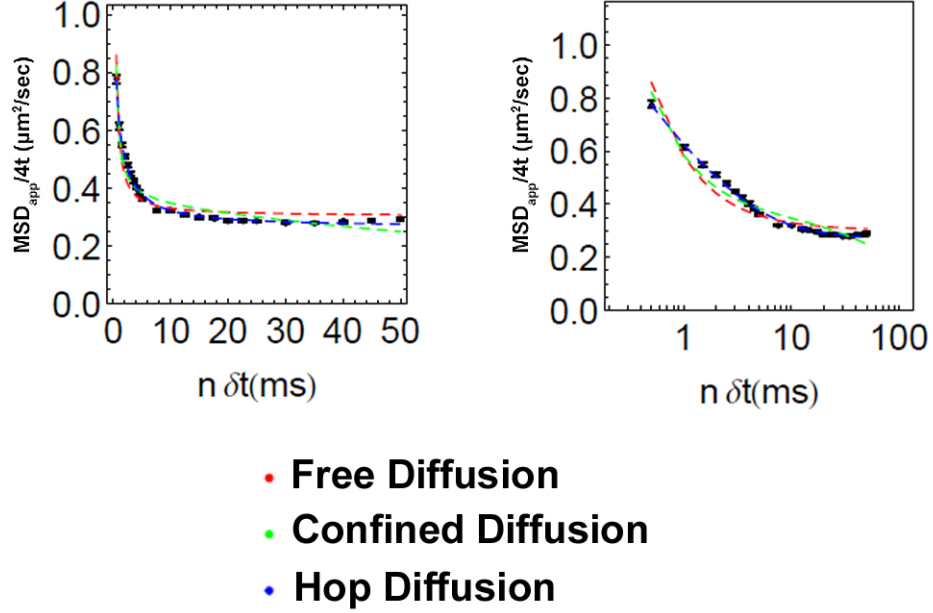


Figure 3.13: Results of the fit of the blur-corrected $MSD_{app}(t)/4t$ values for the example track in Fig.3.11. The dashed lines are the fits to the data of the different diffusion models adopted in this work.

Table 3.3: Results for the fitting of $MSD_{app}(t)/4t$ curves for the example track in (Fig.3.11a with the different diffusion models)

Fit Model		Free: $D + \frac{\delta^2}{2nt_{lag}}$	
Relative Likelihood	Fit Quality	D ($\mu m^2/s$)	δ (nm)
$2 * 10^{-15}$	Good	0.3 ± 0.1	24 ± 1

Fit Model		Confined: $D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta^2}{2nt_{lag}}$			
Relative Likelihood	Fit Quality	D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
$2 * 10^{-13}$	Good	0.35 ± 0.01	66 ± 17	525 ± 70	22 ± 1

Fit Model		“Hop” diffusion: $D_M + D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta^2}{2nt_{lag}}$				
Relative Likelihood	Fit Quality	D_M ($\mu m^2/s$)	D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
1	Good	0.26 ± 0.01	0.39 ± 0.06	1.2 ± 0.2	75 ± 8	14 ± 2

3.2.3 Summary of analysis results

One of the main strengths of SPT is the possibility of analyzing molecular dynamics in a sample with single particle specificity. However, this task can be quite difficult, especially when dealing with tens of tracks, as in the present case. It is therefore necessary to make a choice on how to best summarize the results obtained. One way of achieving this is by running the analysis and model fitting for all the collected gold nanoparticle tracks, as in the previous subsection, and segment them by the most likely diffusion mode. In the pie charts in Fig. 3.14 we report both the results for the MSD and the Step Distribution Analysis, as we stress the fact that, in principle, both methods are equivalent, and choosing between one or the other is completely arbitrary. A possible discrimination method for excluding tracks from the summary lies in the “Fit Quality” assessment. In the pie charts on the right of Fig. 3.14, we excluded those tracks for which no fit model converged. Contrary to what has been reported for the low framerate iSCAT-SPT and STED-FCS experiments, we notice that a majority of the fits fall into the hop diffusion category.

Another way of choosing the most representative model for a number of tracks collected by SPT is by studying the result of the average for all the track data. This procedure is very widely adopted, and offers the advantage of increasing the sample size for each timepoint, thereby reducing the statistical error. The results of such operation, alongside the model fitting, for the Step Distribution Analysis and the $MSD_{app}(t)/4t$ curves are shown in Fig. 3.15 and 3.16.

In order to offer a reference on how the models with $D > 0$ behave at longer times in the $MSD/4t$ vs. t visualization we have elected to use, a fourth model was added, which is dubbed “immobile particle”, equivalent to the Free diffusion case (Eq.2.14), with $D = 0$:

$$D_{app}(nt_{lag}) = \frac{\delta_{x^2+y^2}^2}{2nt_{lag}} \quad (3.2)$$

where δ represents the localization error in two dimensions. A summary of the goodness-of-fit calculated parameters, for both data analysis methods, is given in Tables 3.4 and 3.5.

From these values, we can immediately appreciate how the Reduced χ^2 does not offer a very reliable indicator of statistical significance for the models. In fact, the variation of this value across the Free, Confined and Hop diffusion model is minimal. In the same way, the Fit Quality metric, derived from the convergence of all fit parameters, is not helpful in choosing the most appropriate fitting model. Conversely,

40nm Gold Nanoparticles on SLB

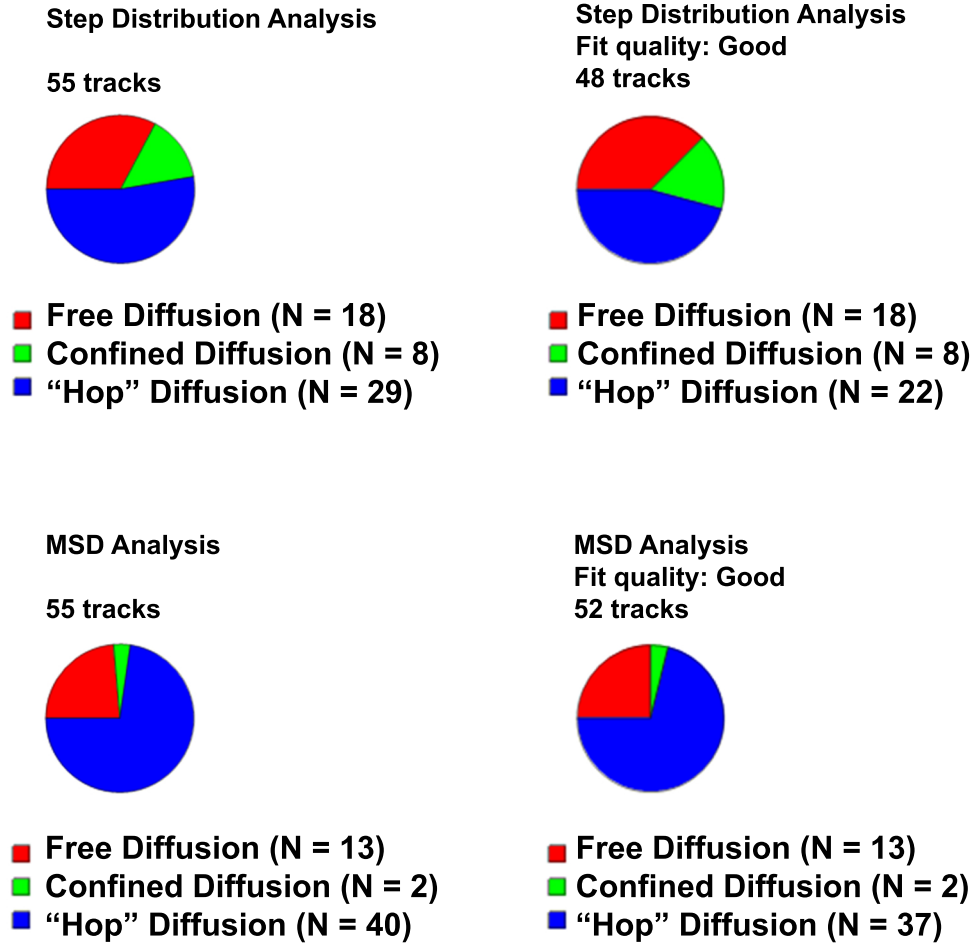


Figure 3.14: Pie charts summarizing the diffusion models fitting results for the $MSD_{app}(t)/4t$ and Step Distribution analysis for 40 nm Gold Nanoparticles-tagged biotinylated lipids diffusing on an SLB (Fig.3.10). On the left: charts for the Step Distribution (top) and $MSD_{app}(t)/4t$ analysis, where the tracks have been categorized by selecting the fitting model with the highest Relative Likelihood (calculated from the BIC). On the right: the same charts as the left, with the added condition that the most likely model should also satisfy the condition, for each of the parameters, that $p < 0.34$.

the BIC and BIC-derived Relative Likelihood offer a more immediate outlook on which model should be chosen to best represent the gold nanoparticle diffusion. Both analysis method therefore agree on the Hop Diffusion model being the most suited to describe this experiment. This is not an obvious conclusion, as the charts in Fig. 3.14 only gave a slim margin of preference for Hop diffusion in the Step Distribution Analysis for each singular track. The results for the data fits, alongside the plots, for

3.2. FAST SAMPLING OF GOLD NANOPARTICLE DIFFUSION

Table 3.4: Goodness of fit parameters for the Step Distribution Analysis curves in Fig.??

Fit Model	Reduced χ^2	BIC	Relative Likelihood	Fit Quality
Immobile	0.6	8.0	$4 * 10^{-33}$	good
Free Diffusion	0.9988	-123.9	$2 * 10^{-4}$	good
Confined Diffusion	0.9988	-120.7	$4 * 10^{-5}$	bad
Hop Diffusion	0.996	-141.04	1	good

Table 3.5: Goodness of fit parameters for the $MSD_{app}(t)/4t$ curves in Fig.??

Fit Model	Reduced χ^2	BIC	Relative Likelihood	Fit Quality
Immobile	0.57	8.3	$2 * 10^{-33}$	good
Free Diffusion	0.997	-103.6	$4 * 10^{-9}$	good
Confined Diffusion	0.9988	-121.3	$3 * 10^{-5}$	good
Hop Diffusion	0.99602	-142.3	1	good

each model are given in the following pages.

From the graphs in Fig. 3.15 and 3.16, it is possible to visualize why the Confined diffusion model does not fit well to the average curve. In fact, this model tends to zero when t becomes large, whereas the values tend, instead, to plateau to a non-zero value. This also excludes, obviously, the immobile particle model. The two remaining models in play are the Hop diffusion and the Free diffusion model, which, as we saw before (Fig. 3.14), are indeed the most representative. By restricting the conclusions to the Hop diffusion model parameters (which is anyway the most likely model to fit the average), in both analysis methods, we find two different diffusion coefficients: the “macroscopic” D_M , corresponding to the Brownian-like dynamic outside the confinement areas, and a “microscopic” one D_μ . Given the expression reported in the Tables 3.6 and 3.7, for large t , the expression for the Hop Diffusion tends to “level off” to the value of D_M . In both analyses, these values are quite close to the values of the Diffusion coefficient obtained for gold nanoparticle-tagged DSPE-PEG(2000)-Biotin that we analysed in the previous section, although obtained with a different bilayer composition.

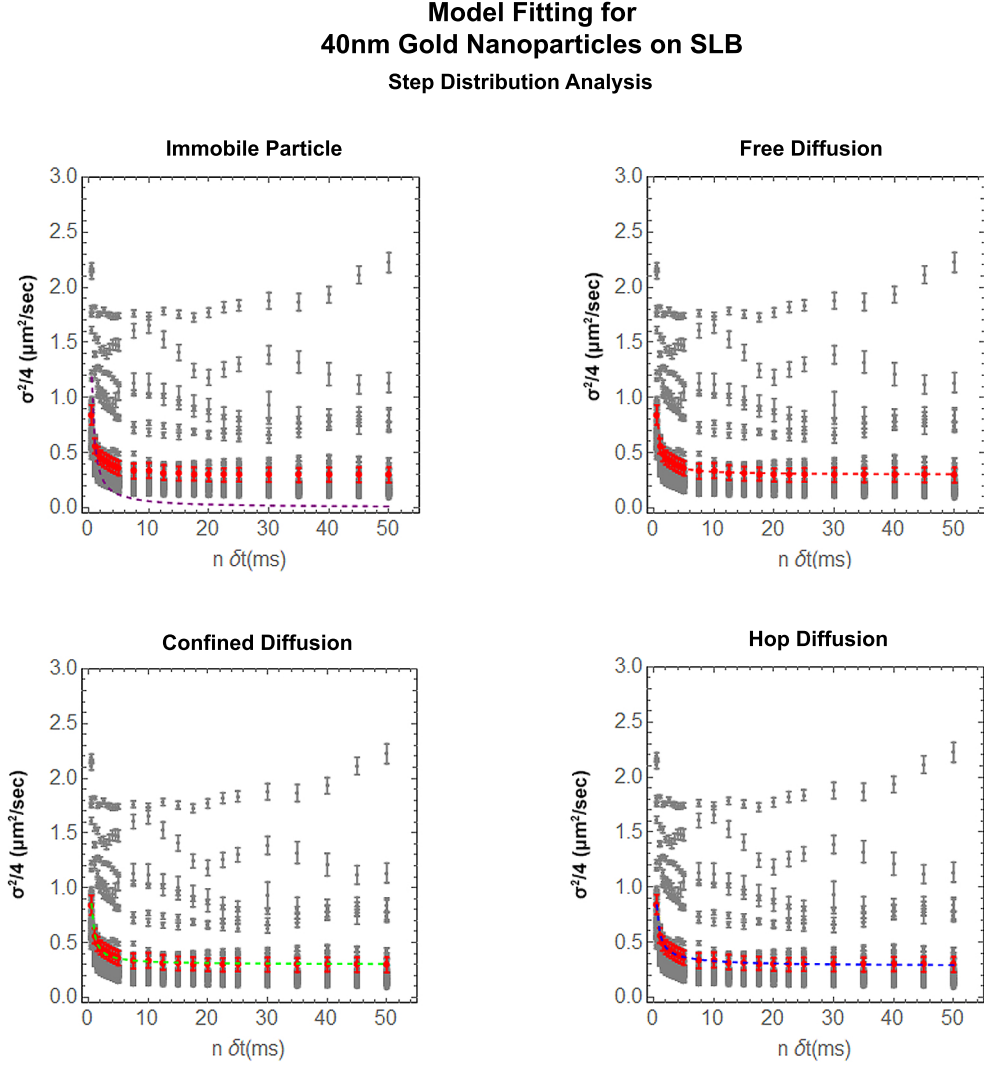


Figure 3.15: Results of the fitting of the diffusion models (Eq. 2.14, 2.15 and 2.16) to the average of camera blur-corrected $\sigma^2(t)/4$ values extracted from the Step Distribution Analysis for all the 40 nm gold nanoparticle-tagged phospholipid tracks.

3.2. FAST SAMPLING OF GOLD NANOPARTICLE DIFFUSION

Table 3.6: Fitting parameters for the average Step Distribution Analysis curve to the different diffusion models (Fig.3.15).

Immobile $\frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$	Free Diffusion: $D + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$	
delta (nm)	D ($\mu m^2/s$)	δ (nm)
34 ± 3	0.300 ± 0.003	23.5 ± 0.3

Confined Diffusion $D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$			
D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
0.3 ± 0.006	$189 \pm 6 * 10^7$	$26085 \pm 4 * 10^6$	23.5 ± 0.4

Hop Diffusion: $D_M + D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$				
D_M ($\mu m^2/s$)	D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
0.28 ± 0.006	0.06 ± 0.01	4 ± 2	14 ± 2	22.3 ± 0.4

Table 3.7: Fitting parameters for the average $MSD_{app}(t)/4t$ curve to the different diffusion models (Fig.3.16).

Immobile $\frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$	Free Diffusion: $D + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$	
delta (nm)	D ($\mu m^2/s$)	δ (nm)
34 ± 3	0.300 ± 0.005	22.3 ± 0.5

Confined Diffusion $D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$			
D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
0.329 ± 0.006	111 ± 21	662 ± 62	21.2 ± 0.4

Hop Diffusion: $D_M + D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$				
D_M ($\mu m^2/s$)	D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
0.273 ± 0.005	0.11 ± 0.02	3 ± 1	19 ± 1	19.7 ± 0.5

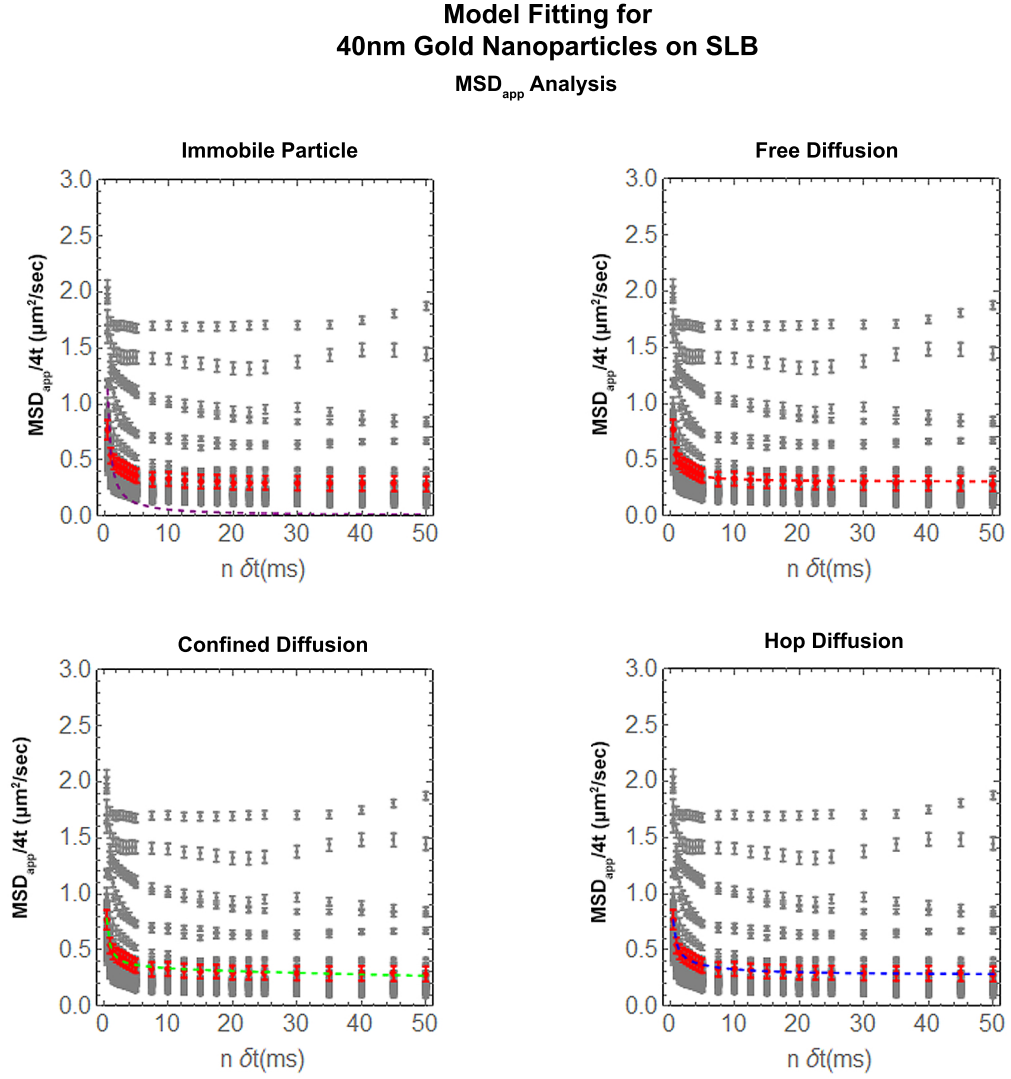


Figure 3.16: Results of the fitting of the diffusion models (Eq. 2.14, 2.15 and 2.16) to the average of camera blur-corrected $MSD_{app}(t)/4t$ values extracted for all the 40 nm gold nanoparticle tracks.

3.2.4 The most likely model for Gold-tagged lipids diffusing on SLB

In the top sections Fig.3.17 and 3.18 we report the detailed graphs of the most likely model for the diffusion of 40 nm gold nanoparticle-tagged phospholipids on the SLB surface, detected at 2 kHz. From the original curves (gray) collected for both analysis methods, we could see that while a large majority of the curves is clustered around low values of $MSD_{app}(t)/4t$, some of the trajectories appear to be diffusing much faster than the average. In fact, some of the curves, in the right part of the graph, reach values for D_M (following the adopted parameter naming convention for hop diffusion) up to until ~ 6 times as much as the values calculated for the original curve ($0.28 \pm 0.01 \mu m^2/s$ for Step Distribution Analysis, and $0.273 \pm 0.005 \mu m^2/s$ for the MSD_{app}). The interpretation we can give of this phenomenon is that while the majority of the gold nanoparticles cross-linked several lipids, thereby diffusing at a slower rate [64, 65], a minority of them did not, thus being able to diffuse faster. The reason behind this may be due to a local lower concentration of biotinylated lipids to link. There is also a distinct possibility that the presence of defects on the SLB surface could concur to slowing down the particles diffusing on the lipid surface.

Alongside the simple fits, in the bottom parts of the following figures we also present the same data, but where we have subtracted the localization error from the values of $MSD_{app}(t)/4t$ and $\sigma^2(t)/4$. We achieved that by taking the values of the parameter $\delta_{x^2+y^2}$, as estimated by the curve fitting operation, and obtaining a value for “ $D_{real}(t)$ ” according to the operation:

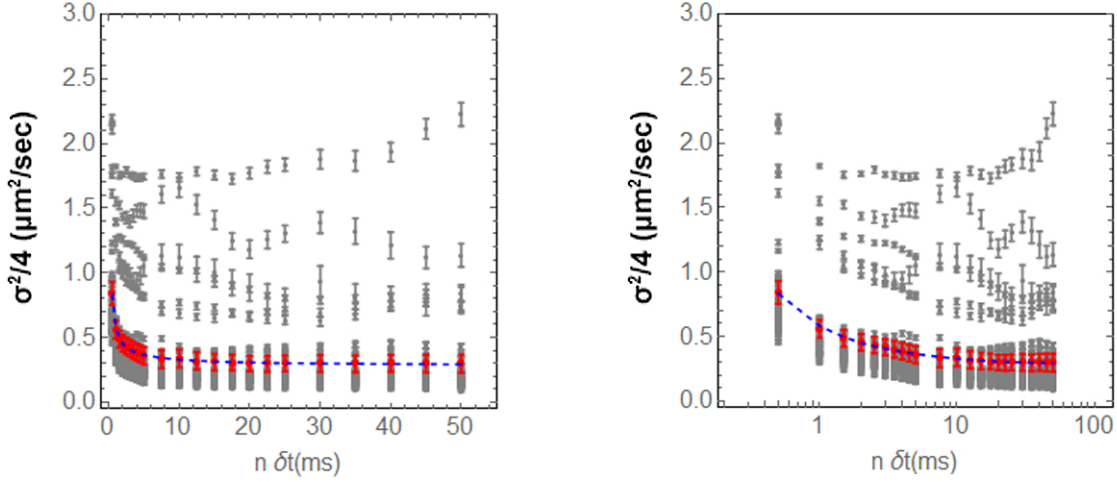
$$D_{real}(t) = \frac{\sigma^2 t - 2\delta^2}{4t} \text{ and } D_{real}(t) = \frac{MSD_{app}t - 2\delta^2}{4t} \quad (3.3)$$

obtained through simple algebra, from Eq. 2.14, 2.15 and 2.16. This operation was applied to the data extracted from all tracks, from the average values and the fitted curve as well. Through this operation, it is possible to calculate the values for the “real”, unaffected by localization noise $\sigma^2(t)/4$ and $MSD_{app}(t)/4t$. In fact, we can appreciate how the shape of the curves, especially in the first few points, does not tend to large values of D anymore, agreeing with the point made in section 1.2.5. However, since the localization noise subtracted is deduced from the average, some of the slowest curves (lower part of the graph), tend to smaller values in the first few points of the graph as compared to the later ones. This may be due to the fact that the faster outliers tend to shift the δ parameter towards higher values, penalizing

slower particles. Since faster particles are more difficult to localize, due to their higher diffusion rate, this is another reasonable analysis outcome.

**Fit of the most likely model (hop diffusion)
for the average curve of
40nm gold-tagged lipids on an SLB**

Step Distribution Analysis



**Localization error subtracted
Step Distribution Analysis**

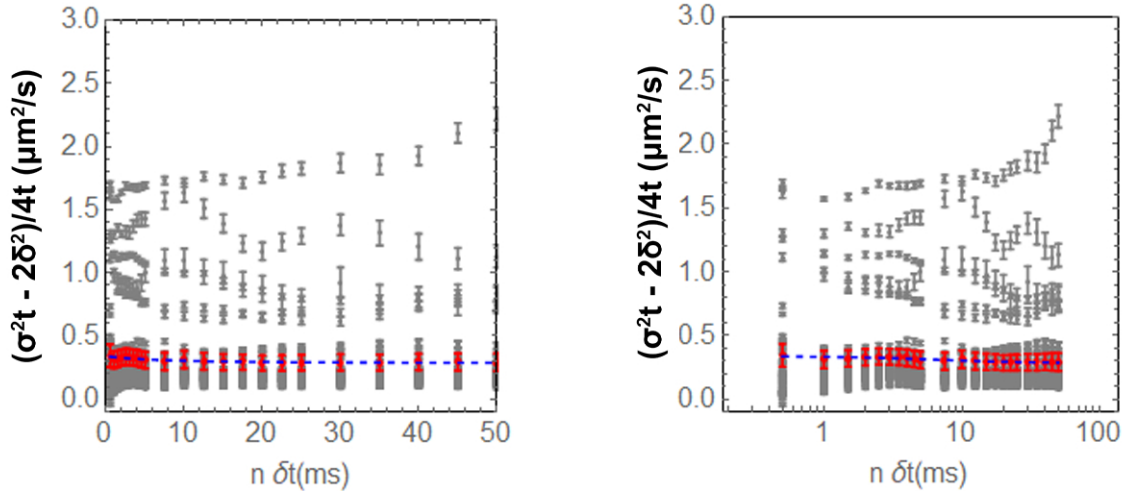


Figure 3.17: Fit of the most likely model, according to Step Distribution Analysis, for 40 nm gold nanoparticle-tagged lipids diffusing on an SLB. (Top) Curve fits for the hop diffusion model, reported in linear (left) and log (right) time scale. The single trajectory data are in gray, the average $\sigma^2(t)/4$ curve is in red, and the fitted curve is in blue. (Bottom) The same data fits from the top plots, in linear (left) and log (right) time scale, where the localization error estimated through the fit has been subtracted.

**Fit of the most likely model (hop diffusion)
for the average curve of
40nm gold-tagged lipids on an SLB**

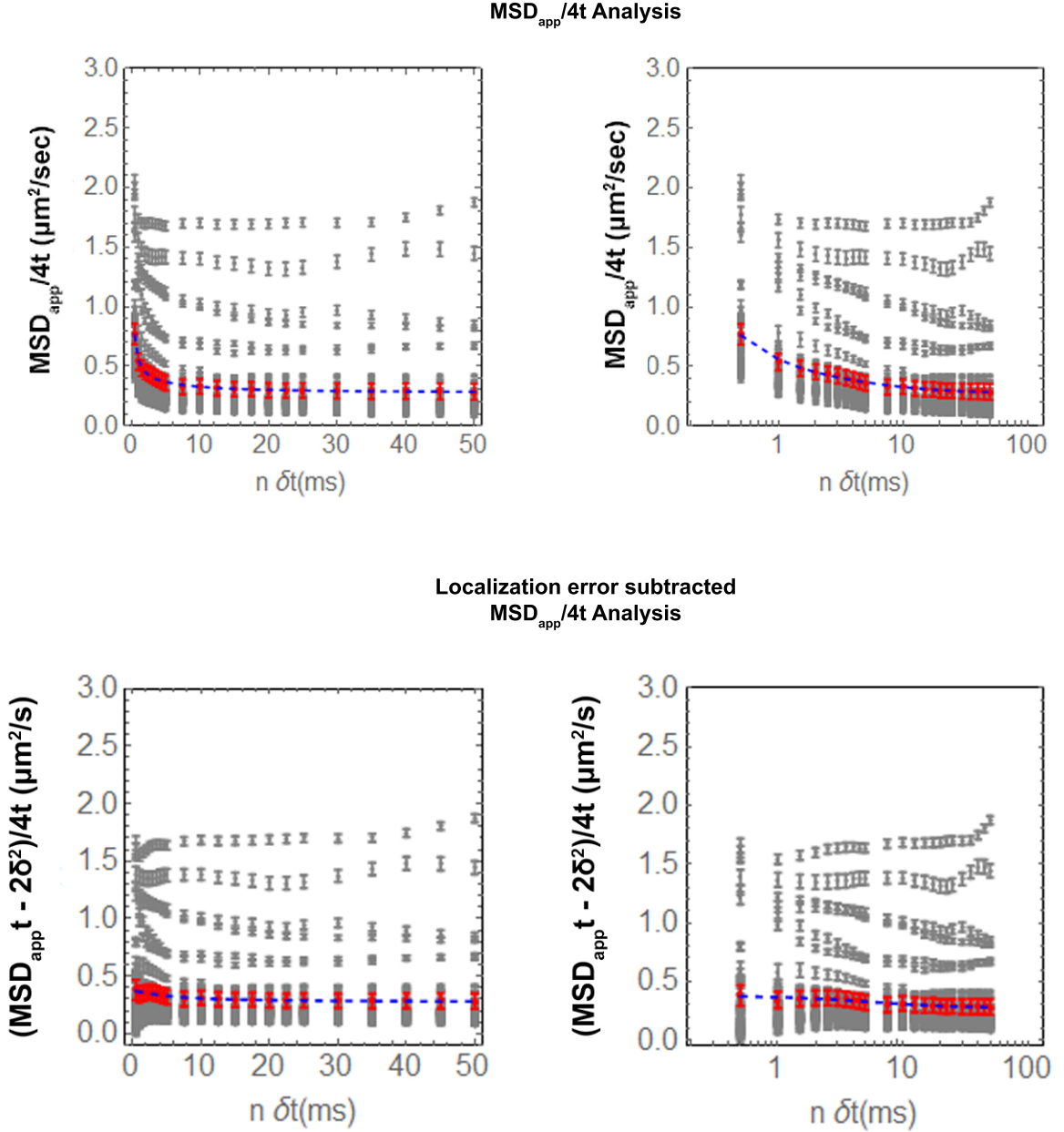


Figure 3.18: Fit of the most likely model, according to $MSD_{app}(t)/4t$ analysis, for 40 nm gold nanoparticle-tagged lipids diffusing on an SLB. (Top) Curve fits for the hop diffusion model, reported in linear (left) and log (right) time scale. The single trajectory data are in gray, the average $MSD_{app}(t)/4t$ curve is in red, and the fitted curve is in blue. (Bottom) The same data fits from the top plots, in linear (left) and log (right) time scale, where the localization error estimated through the fit has been subtracted.

3.3 Conclusions

First of all, we point out how the data presented in this chapter gave us some valuable insights on the use of gold nanoparticles as Single Particle Tracking tags. The SPT data presented in section 3.1 show how the apparent diffusion mode of the gold-tagged lipids is unaffected by either the size or the crosslinking capabilities of the particle. This was confirmed by further point FCS and STED-FCS experiments we executed on the same samples, when the gold nanoparticles were themselves tagged with fluorescent lipids. However, the diffusion rate of the particles is definitely affected by the tag characteristics. This was particularly noticeable as observed by comparing the values of the apparent diffusion coefficient between the gold-tagged and the fluorescent dye-tagged particles obtained by FCS/STED-FCS. We could hypothesize that the observed slow-down in lipid diffusion is due to either the size or the crosslinking potential of the large gold nanoparticle tags, or a combination of these two factors. Which of these effects is most influential, however, cannot be determined by these experiments alone.

These conclusions were refined through the additional high-sampling rate (2kHz) SPT experiments of gold-tagged lipids in a “slower” (i.e., denser) bilayer. We indeed observed an expectedly slower diffusion rate than in the “faster” bilayer previously employed. However, the adoption of faster sampling rates revealed a tendency of the gold-tagging to induce transient confinements, possibly due to a combination of crosslinking of multiple target lipids and defects on the SLB surface. These details could not be resolved at low sampling rates, such as the one adopted in the initial experiments (100Hz), given their short lifetime ($\tau \approx ms$).

Finally, we highlight the application of the devised analysis pipeline, as explained in section 2.2.3 through which we derived new insights on the estimation of the localization error in Single Particle Tracking. In fact, this parameter is usually calculated through the detection of immobilized particles on glass substrates. It was shown how in this way the localization error can be heavily underestimated, given that moving particles will have a lower Signal-to-Noise Ratio than moving particles. Therefore, we propose that localization errors in SPT should be estimated through the curve fittings, for a more precise estimation.

Chapter 4

Protein and Polymer Tags for Single Particle Tracking

In the previous Chapter, we have encountered the difficulty of determining the influence of size and the crosslinking potential of large gold nanoparticle tags on the diffusion dynamics of the target lipids. We now aim at gaining deeper insights in this matter with the tracking of two protein tags (a crosslinking and a strictly monovalent one) using complementary iSCAT-SPT and FCS measurements.

The possibility of detecting single proteins without the aid of large scattering tags [104, 117] is one of the most interesting features of iSCAT microscopy applied to Life Sciences. In this chapter, we initially present the data on the diffusion dynamics for Streptavidin- and Flavidin-tagged lipids on a Supported Lipid Bilayer (SLB).

The difference between these two protein tags lies in their biotin-binding capabilities. In fact, commercially-available Streptavidin can bind up to four biotin molecules, while artificially synthesized Flavidin (courtesy and kind gift of Prof. Howarth's laboratory) only has one viable biotin binding site. The only modifications applied to the original protein are in the amino acid sequence, leaving scattering microscopy-relevant properties (molecular weight, size) identical to original Streptavidin [55, 56]. Using these two protein as tags for iSCAT measurements, we aimed to refine our understanding of the effects of crosslinking on the target molecule dynamics. Since the protein tags were also tagged with fluorescent dyes, we confirmed the results obtained with FCS measurements on the same samples.

Further, the observation of the calibration sample used for the FCS measurements, a simple SLB with the fluorescent lipid analogue DSPE-PEG(2000)-KK114, revealed low scattering intensity moving features. We hypothesized that the origin of such features was due to the 2 kDa PEG linker in the lipid mixture. We verified this hypothesis against a control sample with no PEG linkers, and proceeded to also track

these moving particles. While the analysis of SPT data on PEG-ylated lipids proved of difficult interpretation, future research in this direction might result in the adoption of smaller tags with absolute specificity to track lipid diffusion dynamics.

4.1 Streptavidin- and Flavidin-tagged lipids diffusing on a model membrane

4.1.1 Tags description and tracking

Streptavidin is a tetrameric protein first isolated from the bacterium *Streptomyces avidinii*. The main feature of Streptavidin is its high binding affinity for biotin (also known as vitamin B7), with dissociation constant of $K_d = 10^{-14}$ M, making it one of the strongest naturally-occurring non-covalent bonds known [153]. Amongst its favourable characteristics are high thermal stability and great resistance to pH changes, surfactants and detergents [56]. Compared to other protein-based tagging methods, such as Halo-tag and SNAP-tag, Streptavidin tagging presents the advantage of being more resistant to denaturation [56]. Another reason for its popularity for cellular labelling mainly lies in the fact that biotinylation is a process that rarely disrupts the target molecule behaviour, given how small the biotin molecule is. Furthermore, biotin can nowadays be targeted very precisely to many kinds of molecules, and several biotin and Streptavidin conjugates are often readily available commercially. Finally, Streptavidin also has very low non-specific binding [56].

Despite all these advantages, wild-type Streptavidin also carries the main drawback of being tetravalent. In fact, the four monomers that make up the complete protein all have equal biotin-binding capabilities. Therefore, Streptavidin can bind up to four biotin targets. Crosslinking can be overlooked, and can sometimes even be beneficial, in the case of labelling for imaging. However, this is not the case for single particle tracking experiments, where strictly monovalent tags are always preferable. Several attempts have been made to obtain single biotin-binding Streptavidin variants. A few successful variants have been obtained by modifying the interface between the subunits, a process that unfortunately leads to the decrease of biotin affinity [55, 154].

The strategy adopted in Prof. Mark Howarth's laboratory is to modify the binding sites of the single subunits by aminoacid substitutions, to either enhance or cancel their binding properties. The missing hydrogen bond capabilities and steric hindrances of the binding site of the inactive subunits forbids biotin binding. These

subunits are then left to bind with enhanced binding ones, which in turn have a slower dissociation rate from biotin than wild type streptavidin subunits. Thus, a mixture of variants which can bind to a different number of biotin molecules is obtained. The single-binding ones can then be separated by column purification [55]. The biotin binding subunit also presents up to four solvent-exposed amine groups for binding NHS-ester fluorescent dyes (in our case, Abberior STAR 635P). The full name for this tag is thus “mono-trapflavidin”, which we shorten to “Flavidin” in the following to simplify the notation (see also Materials and Methods) [58].

The Streptavidin solution we used was purchased as an Abberior STAR 635P fluorescent dye conjugate (Abberior GmbH). The Flavidin solution was provided courtesy of Prof. Mark Howarth’s laboratory (Dept. of Biochemistry, University of Oxford) after conjugation with the same fluorescent dye. Both these proteins can be detected in iSCAT microscopy (inserts in Fig. 4.1a and b), and we used them to tag biotinylated, saturated lipids on a Supported Lipid Bilayer. The bilayer composition used for these experiments was: 74.5 mol% DOPC, 25 mol% cholesterol, and 0.5 mol% DSPE-PEG(2000)-biotin, for a total lipid concentration of 1mg/ml in a 50:50 methanol:chloroform solution. A small quantity (equivalent to 0.01 mol%) of DPPE-Atto488, a green fluorescent lipid analogue, was added to the bilayer mixture, in order to detect the bilayer using the iSCAT microscope fluorescent channel. The bilayer was tagged with the Streptavidin and Flavidin solutions, diluted of a factor of 20 compared to the starting concentrations. This led to an adequately sparse particle density, which allowed tracking. Movies were collected at 2 kHz sampling frequency, after illuminating the sample with 7.3mW laser power ($\lambda = 660$ nm), with a 2 s observation window. The detected tracks are shown in Fig.4.1.

We calculated the $MSD_{app}(t)/4t$ and the $\sigma^2(t)/4$ from the Step Distribution Analysis for each of the tracks collected for both Streptavidin and Flavidin (see section 2.2.3). After the data were corrected for camera blur, we proceeded with the quantitative analysis of these values through model fitting. Therefore, the values obtained were fitted using the three diffusion models presented in the Materials and Methods chapter (Eq. 2.14, 2.15 and 2.16). Each track is subsequently “assigned” to a specific model, based on the highest Relative Likelihood, as calculated from the Bayesian Information Criterion. The results of this segmentation operation are visualized in Fig. 4.2 and Fig. 04.3, for Streptavidin and Flavidin respectively.

Contrary to what we observed for the 40nm gold nanoparticle tagging, it seems that the dynamics of protein-tagged lipids seem to be described better by a free, unrestricted diffusion model. This result can be expected given that, even for the multiva-

4.1. STREPTAVIDIN- AND FLAVIDIN-TAGGED LIPIDS DIFFUSING ON A MODEL MEMBRANE

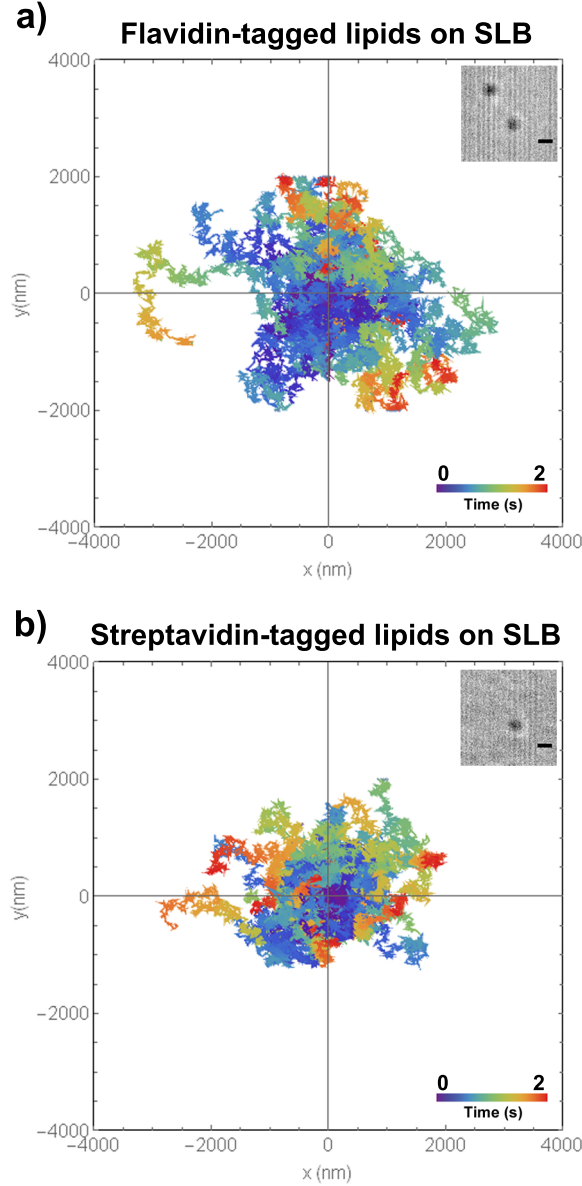


Figure 4.1: Detected trajectories for Flavidin- (a) and Streptavidin-tagged lipids (b) diffusing on an SLB. The tracks are remapped to start at (0;0) coordinate, and go from blue ($t=0$) to red ($t=2$ s). In the insets, the images of a Flavidin (a) and Streptavidin (b), visualized at the imaging conditions exposed in the text (Scale bars 500nm). Amongst all of the detected tracks, only the ones longer than 500 positions were selected. For Flavidin, the number of tracks is $N = 44$, while for Streptavidin $N = 50$.

lent Streptavidin, the crosslinking potential is smaller compared to gold nanoparticles. It is peculiar how the Step Distribution Analysis seems to assign a larger majority of the particles to the free diffusion model in both cases, while the $MSD_{app}(t)/4t$ charts are less defined in that sense. Since there is no *a priori* reason to prefer either of the analysis methods, we here report both outputs.

Streptavidin on SLB

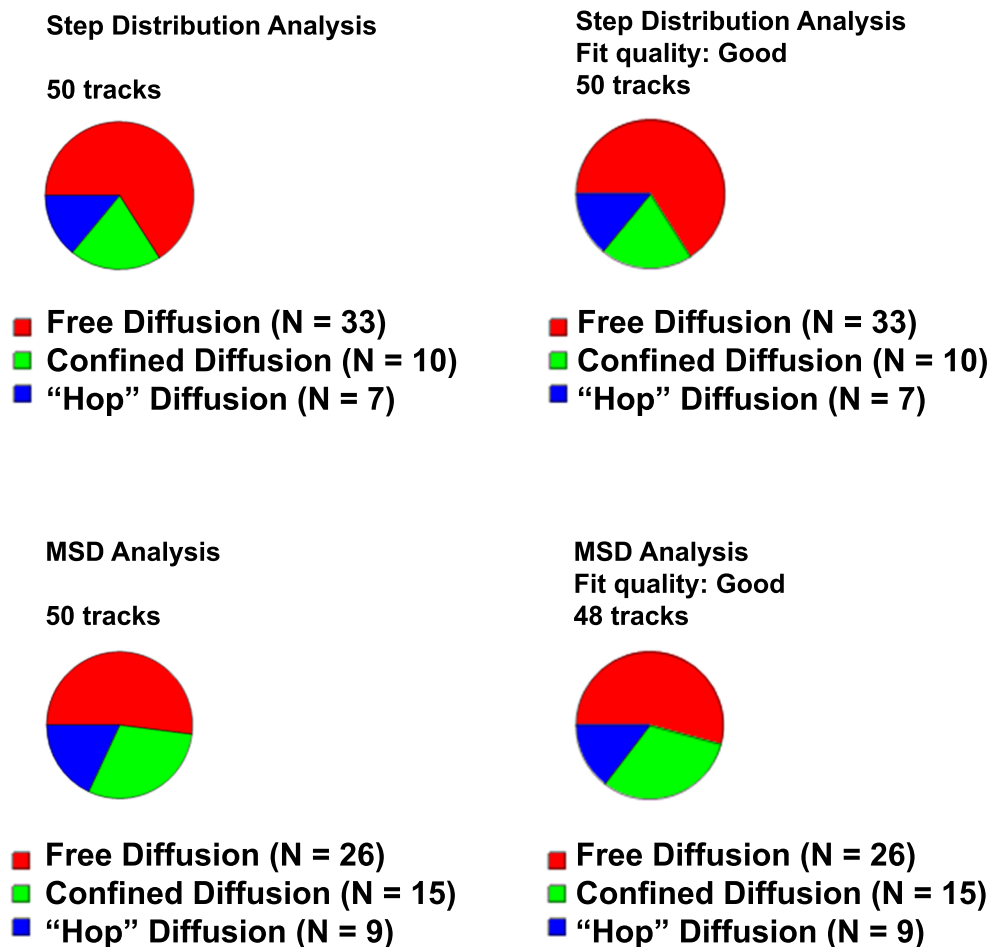


Figure 4.2: Pie charts summarizing the diffusion models fitting results for the $MSD_{app}(t)/4t$ and Step Distribution analysis for Streptavidin-tagged biotinylated lipids on an SLB. On the left: charts for the Step Distribution (top) and $MSD_{app}(t)/4t$ analysis, where the tracks have been categorized by selecting the fitting model with the highest Relative Likelihood (calculated from the BIC). On the right: the same charts as the left, with the added condition that the most likely model should also satisfy the condition, for each of the parameters, that $p < 0.34$.

4.1. STREPTAVIDIN- AND FLAVIDIN-TAGGED LIPIDS DIFFUSING ON A MODEL MEMBRANE

Flavidin on SLB

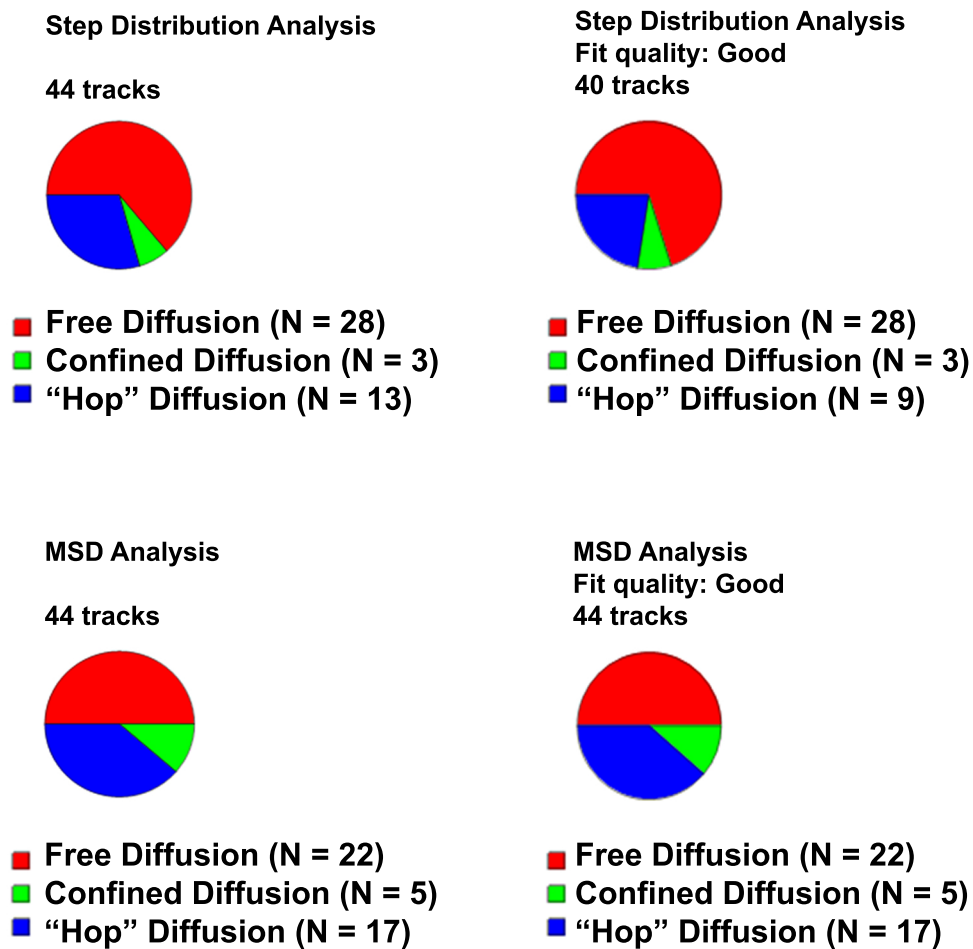


Figure 4.3: Pie charts summarizing the diffusion models fitting results for the $MSD_{app}(t)/4t$ and Step Distribution analysis for Flavidin-tagged biotinylated lipids on an SLB. On the left: charts for the Step Distribution (top) and $MSD_{app}(t)/4t$ analysis, where the tracks have been categorized by selecting the fitting model with the highest Relative Likelihood (calculated from the BIC). On the right: the same charts as the left, with the added condition that the most likely model should also satisfy the condition, for each of the parameters, that $p < 0.34$.

4.1.2 SPT analysis for Streptavidin and Flavidin-tagged lipids

Following the standard procedure for SPT data analysis, the single particle trajectories obtained are analyzed by calculating the Mean Squared Displacements of each, and evaluating the average diffusion behaviour by looking at the ensemble average of these values. The underlying assumption is that, given that we are in the presence of a uniform, at least in principle, supported lipid bilayer, the average values should be a good representation of the particles diffusivity. From the graphs in Fig. 4.4, where we plotted and averaged the MSD values up until 50 ms, we can observe that the particles seem to behave according to a free diffusion model. Therefore, the free diffusion model in Eq. 2.4 was fitted to the data. From this operation we derived that the Flavidin-tagged lipids diffuse faster, with an average rate of $0.82 \pm 0.004 \mu\text{m}^2/\text{s}$, while the Streptavidin-tagged lipids diffusion coefficient is $0.56 \pm 0.005 \mu\text{m}^2/\text{s}$. The significance of these values is discussed further ahead in the text. The particle localization error was estimated, with this model, at $\delta = 37.4 \pm 0.2$ for the Flavidin tags and $\delta = 40 \pm 0.2$ for the Streptavidin tags.

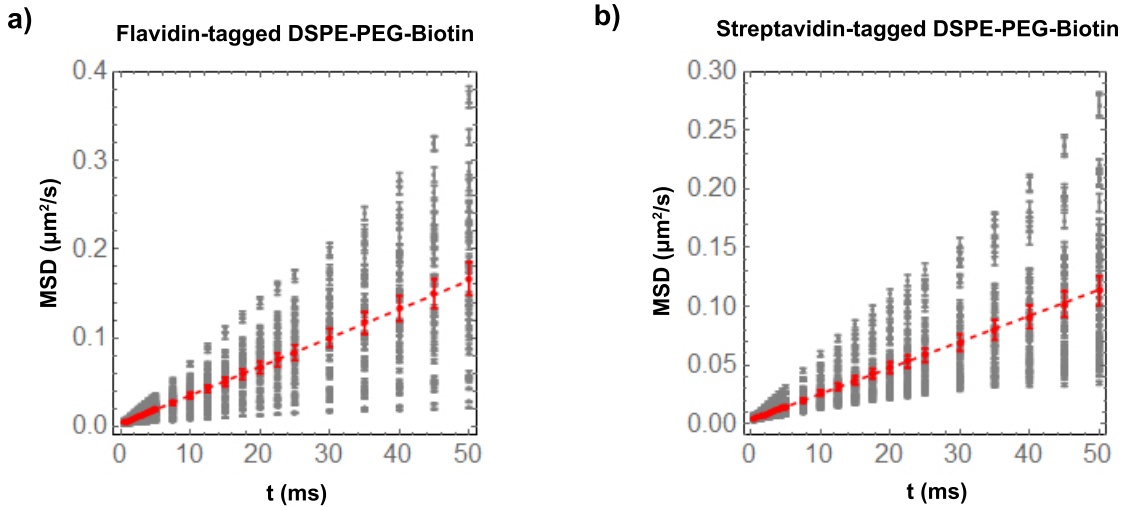


Figure 4.4: MSD vs. t plots of the tracks detected for (a) Flavidin-tagged lipids and (b) Streptavidin tagged lipids. The single trajectories (grey) were used to produce the ensemble average MSD values (red). A free diffusion model (Eq. 2.4) was fitted to the ensemble average data (red dashed line) to obtain the values of diffusion coefficient and localization precision, reported in the text.

The same particle tracks were also analyzed following the revised analysis method we laid out in Section 2.2.3. We therefore analyzed the ensemble averages of the curves obtained with both the $MSD_{app}(t)/4t$ and the Step Distribution methods. We attempted to fit to such data the four models of choice (immobile particle, free,

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confined, and hop diffusion). The fit results are graphically compared in Fig. 4.5 for Streptavidin-tagged lipids, and in Fig. 4.6 for Flavidin-tagged ones. In these graphs, as expected, the immobile particle model clearly does not fit the averaged data at all, whereas the diffusing particle models are not clearly distinguishable from one another. In order to discern which is the most appropriate model we calculated the statistical goodness-of-fit parameters, as we already did in the gold nanoparticle case (Chapter 3). These are reported in the Tables 4.1, 4.2, 4.3 and 4.4. Unsurprisingly, the best-fitting model for both tagging methods is the free diffusion mode, for the $MSD_{app}(t)/4t$ and Step Distribution Analysis alike. Once again, we remark how the reduced χ^2 is insufficient to appreciate the difference in the fit goodness between these models, whereas the BIC gives a more precise way of discerning between them.

Contrary to the analysis performed on the 40nm gold nanoparticles, we can here better appreciate the necessity for adopting a condition on the convergence of the model fits, which we dubbed “Fit Quality”. We recall that this arbitrary condition states that the p-values calculated for each of the parameters (as supplied by Wolfram Mathematica) should satisfy the relation $p < 0.34$. This is equivalent to saying that the probability of the particular parameter being equal to the stated value is $> 2\sigma$. In practical terms, this means that the confidence interval for each parameter cannot be too large. By using this condition, we can *a priori* reject models which have a significant Relative Likelihood but whose parameters did not converge well.

In the following pages, we report the single model fits, alongside the parameters both for Streptavidin-tagged phospholipids and Flavidin-tagged phospholipids, using both analysis methods. From the comparison of the values for the diffusion coefficients reported in the Tables 4.1, 4.2, 4.3 and 4.4, we notice that the biotinylated phospholipids tagged with the monovalent Flavidin diffuse ~ 1.6 times faster than the ones tagged with the tetravalent Streptavidin. In fact, the parameters extracted from the fit of the free diffusion model tell us that the apparent diffusion coefficient D_{app} for the Streptavidin tags is $0.58 \pm 0.05 \mu m^2/s$, obtained via the Step Distribution Analysis, and $0.555 \pm 0.005 \mu m^2/s$, obtained fitting the $MSD_{app}(t)/4t$ values. On the other hand, the same values in the case of Flavidin tagging are $0.87 \pm 0.01 \mu m^2/s$ and $0.81 \pm 0.007 \mu m^2/s$, calculated from the Step Distribution Analysis and $MSD_{app}(t)/4t$ fitting respectively.

The difference between the lipids tagged with the two different proteins could be attributed to the crosslinking of multiple biotinylated lipids by the Streptavidin tags. However short-lived these additional bindings are, they evidently slow down the diffusion of the tagged lipids significantly. It is notable how the confined diffusion and

the hop diffusion fits, despite not converging well for neither of the samples nor the analysis methods, present values for the D_μ and D_M , respectively, that are similar to the D obtained by the free diffusion model fitting. This can be expected, given that all models tend to level off to a constant value for very long times. Finally, the localization errors δ obtained from these measurements are, unsurprisingly, larger than those obtained for the 40nm gold nanoparticle tags. In effect, estimation for the Streptavidin tag localization error oscillate between $\delta = 37.1 \pm 0.6$ nm (Step Distribution) and $\delta = 37.4 \pm 0.2$ nm ($MSD_{app}(t)/4t$), while for Flavidin the same parameter was estimated as $\delta = 38.5 \pm 0.3$ nm (Step Distribution) or $\delta = 39.8 \pm 0.2$ nm ($MSD_{app}(t)/4t$). The localization error for the 40nm gold nanoparticle tags on the same SLB, instead was calculated as $\delta = 22.3 \pm 0.4$ nm (Step Distribution) and $\delta = 19.7 \pm 0.5$ nm ($MSD_{app}(t)/4t$). This is of course not surprising, given the difference in scattering cross section between gold nanoparticles and the present protein tags.

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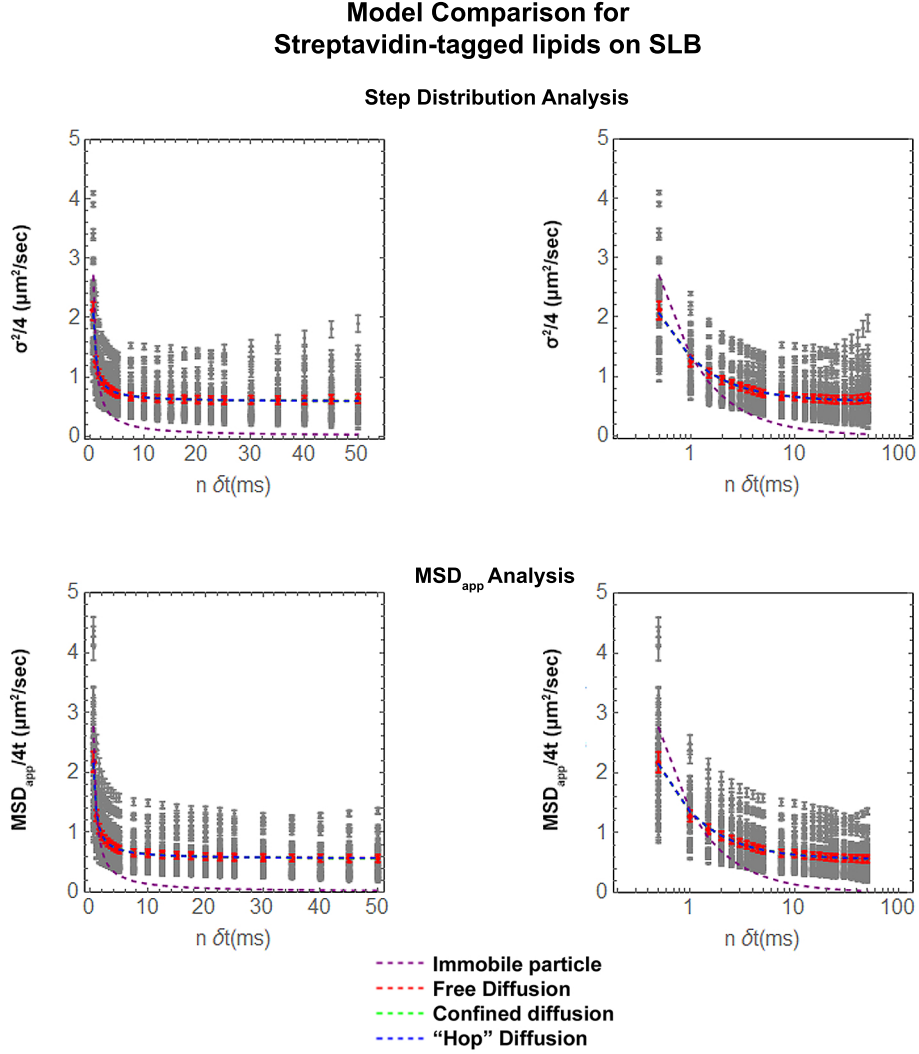


Figure 4.5: Comparison of the fitting models for the average of the ensemble averages of the data for Streptavidin-tagged lipids diffusing on an SLB. (Top) Step Distribution Analysis: original tracks (black), average curve (red), fitted models (dashed lines, colors as in the included legend). Left: time axis in linear scale. Right: time axis in log scale, to accentuate on the first timepoints. (Bottom) Same model fittings and representations, but calculated for the $MSD_{app}(t)/4t$ curves.

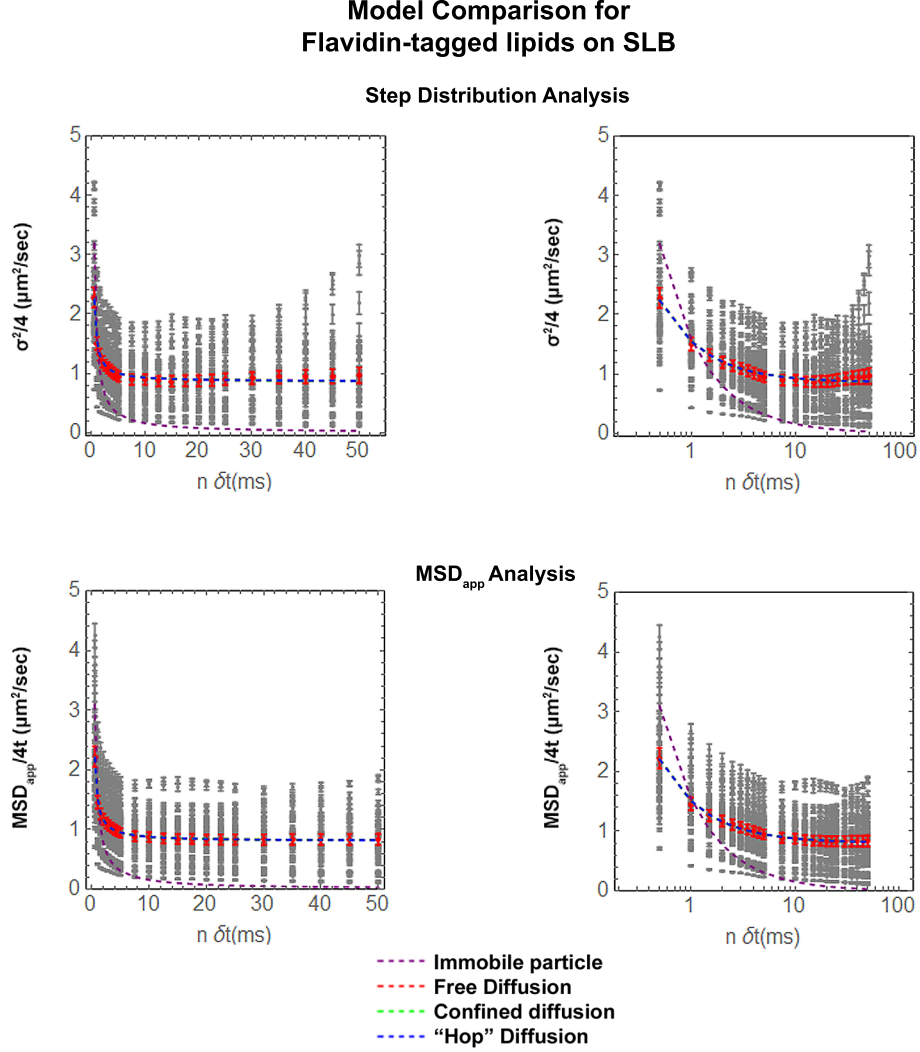


Figure 4.6: Comparison of the fitting models for the average of the ensemble averages of the data for Flavidin-tagged lipids diffusing on an SLB. (Top) Step Distribution Analysis: original tracks (black), average curve (red), fitted models (dashed lines, colors as in the included legend). Left: time axis in linear scale. Right: time axis in log scale, to accentuate on the first timepoints. (Bottom) Same model fittings and representations, but calculated for the $MSD_{app}(t)/4t$ curves.

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Table 4.1: Goodness-of-fit parameters for the Step Distribution Analysis data fits for Streptavidin-tagged phospholipids

Fit Model	Reduced χ^2	BIC	Relative Likelihood	Fit Quality
Immobile	0.68	38.4	$2 * 10^{-31}$	good
Free Diffusion	0.9994	-103	1	good
Confined Diffusion	0.9994	-99.8	0.19	bad
Hop Diffusion	0.9994	-96.56	0.04	bad

Table 4.2: Goodness-of-fit parameters for the $MSD_{app}(t)/4t$ data fits for Streptavidin-tagged phospholipids

Fit Model	Reduced χ^2	BIC	Relative Likelihood	Fit Quality
Immobile	0.7	36.4	$2.2 * 10^{-32}$	good
Free Diffusion	0.9995	-109	1	good
Confined Diffusion	0.9995	-106.23	0.2	bad
Hop Diffusion	0.9995	-102.9	0.04	bad

Table 4.3: Goodness-of-fit parameters for the Step Distribution Analysis data fits for Flavidin-tagged phospholipids

Fit Model	Reduced χ^2	BIC	Relative Likelihood	Fit Quality
Immobile	0.57	56.6	$2 * 10^{-28}$	good
Free Diffusion	0.9985	-70.85	1	good
Confined Diffusion	0.9985	-67.52	0.19	bad
Hop Diffusion	0.9985	-64.3	0.04	bad

Table 4.4: Goodness-of-fit parameters for the $MSD_{app}(t)/4t$ data fits for Flavidin-tagged phospholipids

Fit Model	Reduced χ^2	BIC	Relative Likelihood	Fit Quality
Immobile	0.59	53.7	$2.5 * 10^{-37}$	good
Free Diffusion	0.9998	-115	1	good
Confined Diffusion	0.9997	-111.6	0.19	bad
Hop Diffusion	0.9997	-108.6	0.04	bad

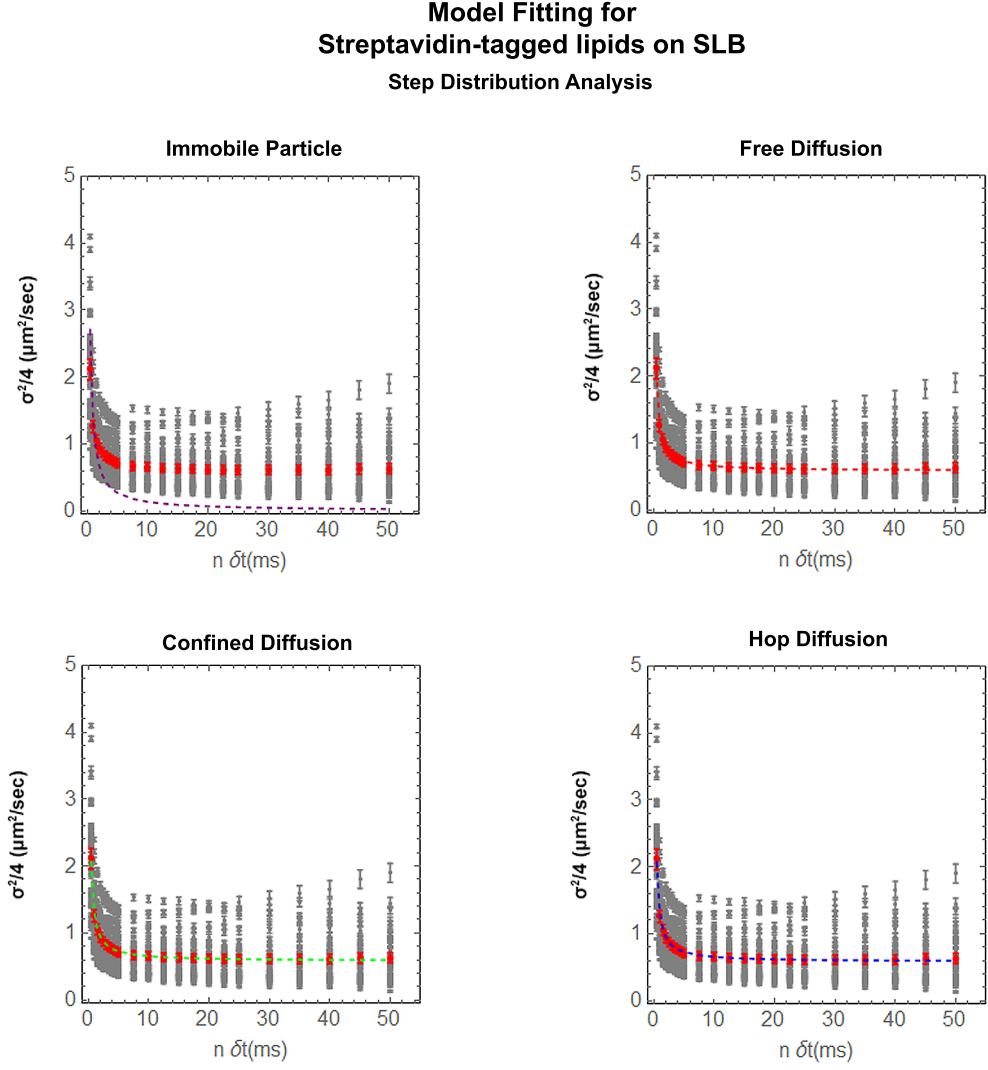


Figure 4.7: Results of the fit of the average of blur-corrected $\sigma^2(t)/4$ values extracted from the Step Distribution Analysis for all the Streptavidin-tagged phospholipid tracks.

4.1. STREPTAVIDIN- AND FLAVIDIN-TAGGED LIPIDS DIFFUSING ON A MODEL MEMBRANE

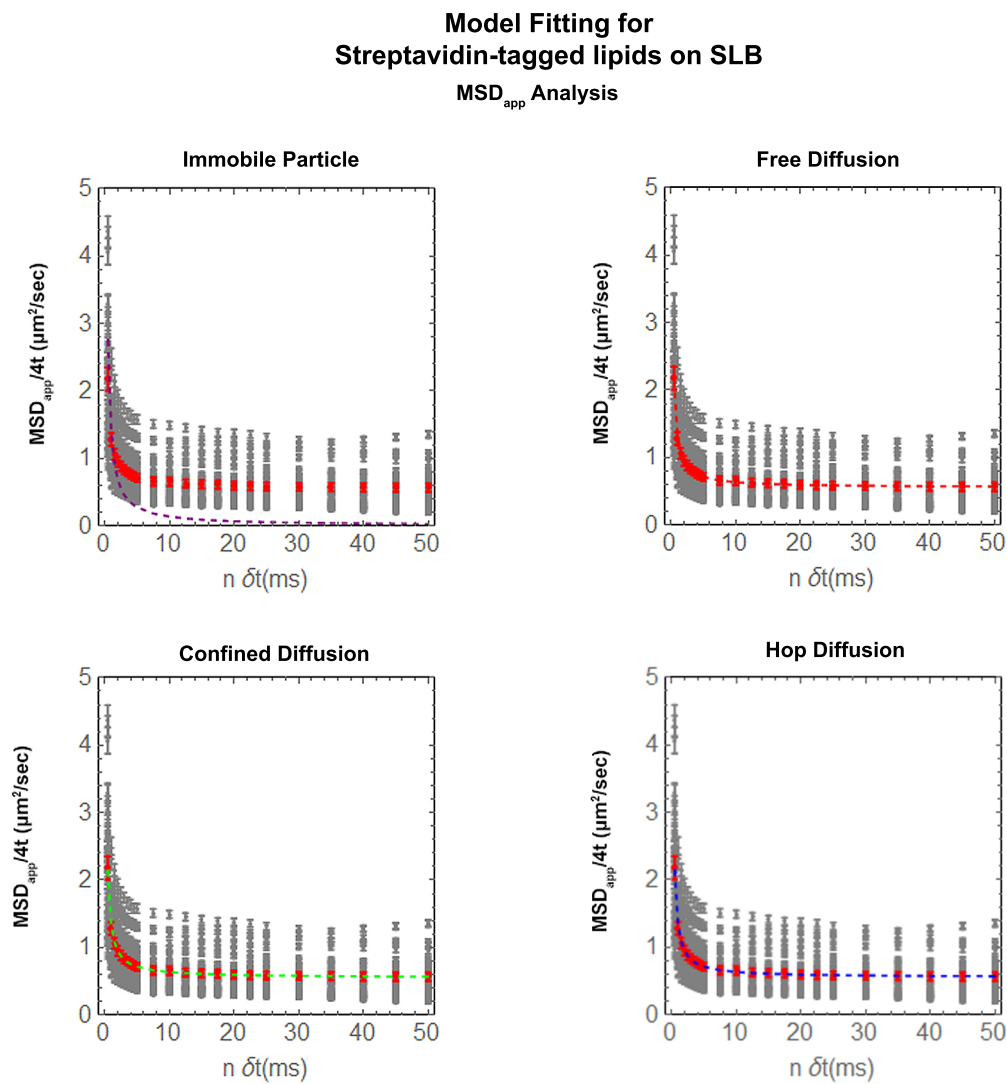


Figure 4.8: Results of the fit of the average of blur-corrected $MSD_{app}(t)/4t$ values for all the Streptavidin-tagged phospholipid tracks.

Table 4.5: Fitting parameters for the average Step Distribution Analysis curve of Streptavidin-tagged phospholipids to the different diffusion models (Fig.4.7).

Immobile $\frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$	Free Diffusion: $D + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$	
delta (nm)	D ($\mu m^2/s$)	δ (nm)
52 ± 4	0.58 ± 0.05	38.5 ± 0.3

Confined Diffusion $D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$			
D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
0.58 ± 0.01	$64338 \pm 5 * 10^6$	$21169 \pm 8 * 10^5$	38.5 ± 0.3

Hop Diffusion: $D_M + D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$				
D_M ($\mu m^2/s$)	D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
0.58 ± 0.05	$4 * 10^{-4} \pm 6$	$7804 \pm 5 * 10^{-7}$	1.6 ± 13006	38.5 ± 0.3

Table 4.6: Fitting parameters for the average $MSD_{app}(t)/4t$ curve of Streptavidin-tagged phospholipids to the different diffusion models (Fig.4.8).

Immobile $\frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$	Free Diffusion: $D + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$	
delta (nm)	D ($\mu m^2/s$)	δ (nm)
52 ± 4	0.555 ± 0.004	39.8 ± 0.2

Confined Diffusion $D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$			
D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
0.555 ± 0.008	25270 ± 734895	12979 ± 188714	40 ± 0.3

Hop Diffusion: $D_M + D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$				
D_M ($\mu m^2/s$)	D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
0.555 ± 0.007	$9 * 10^{-6} \pm 0.4$	0.66 ± 20216	0.2 ± 5322	40 ± 3

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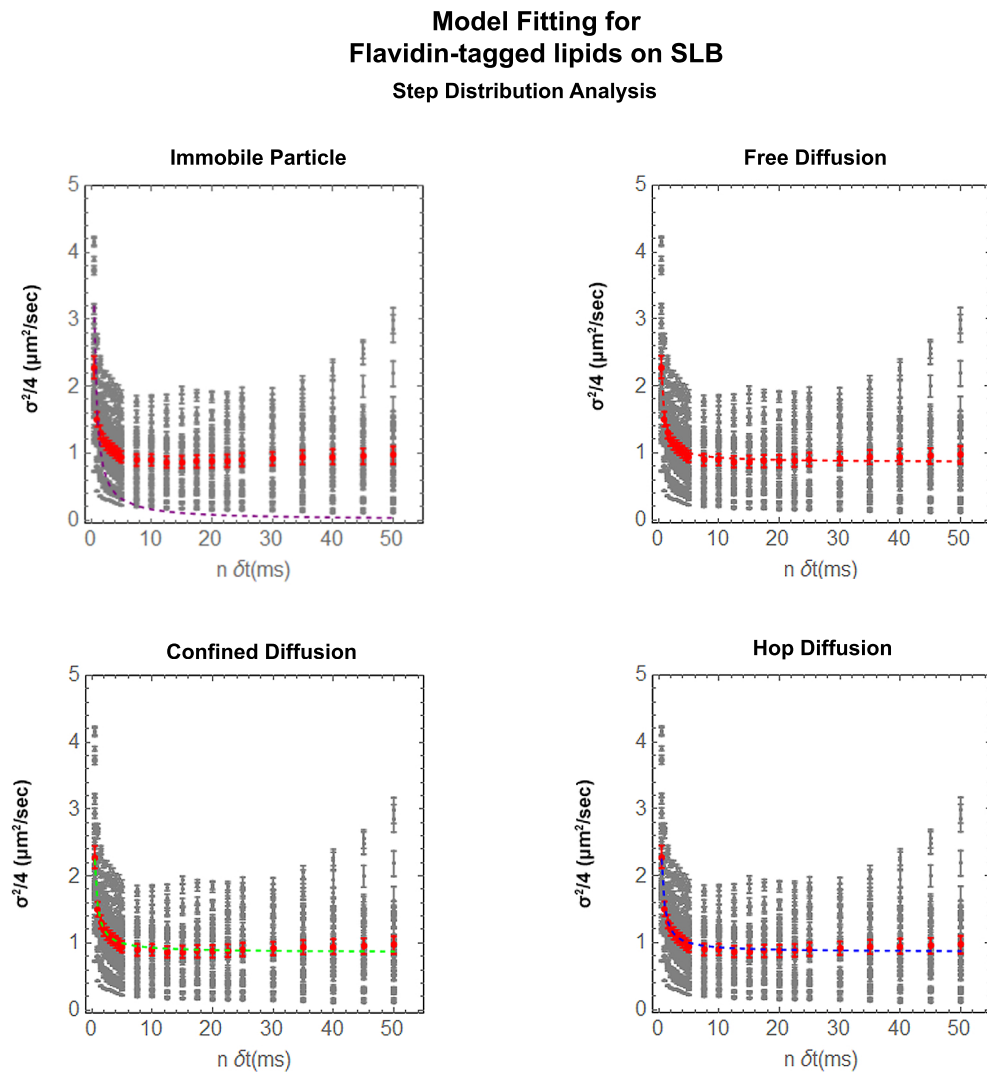


Figure 4.9: Results of the fit of the average of blur-corrected $\sigma^2(t)/4$ values extracted from the Step Distribution Analysis for all the Flavin-tagged phospholipid tracks.

**Model Fitting for
Flavinin-tagged lipids on SLB**
MSD_{app} Analysis

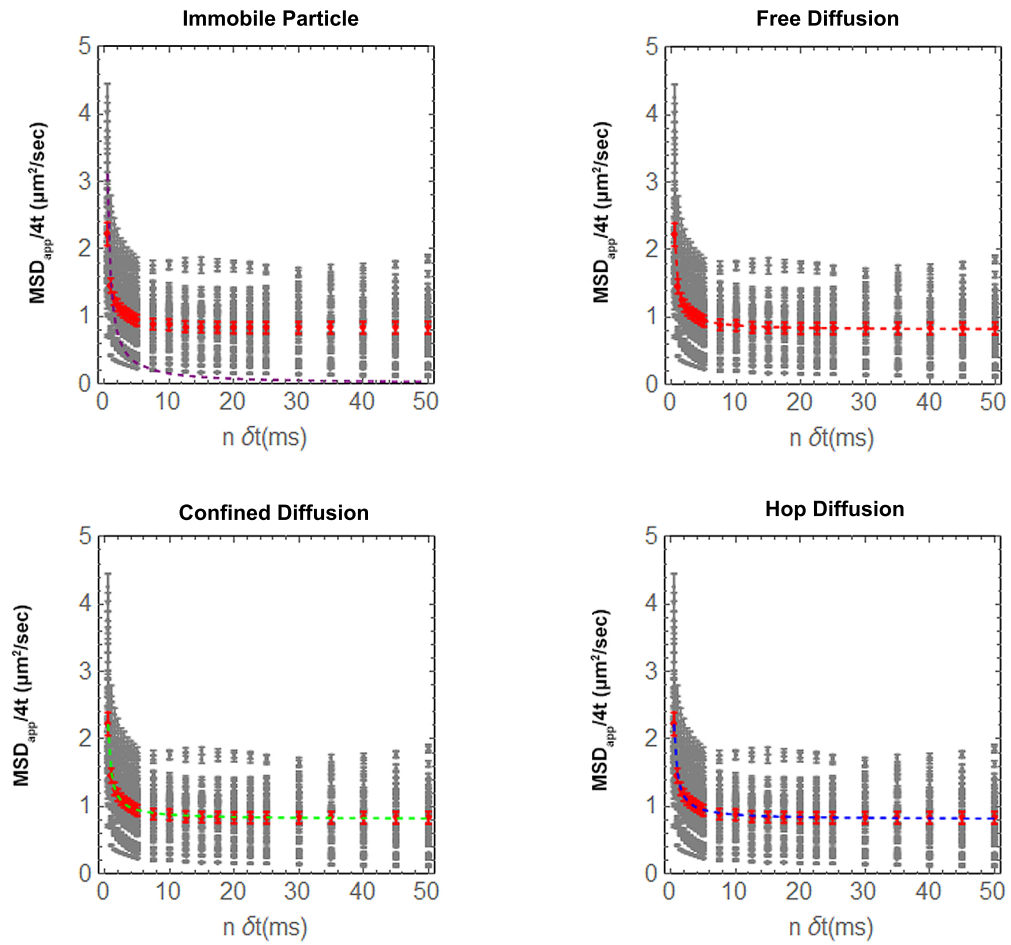


Figure 4.10: RResults of the fit of the average of blur-corrected $MSD_{app}(t)/4t$ values for all the Flavinin-tagged phospholipid tracks.

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Table 4.7: Fitting parameters for the average Step Distribution Analysis curve of Flavidin-tagged phospholipids to the different diffusion models (Fig.4.9).

Immobile $\frac{\delta_{x^2+y^2}^2}{2nt_{lag}}$	Free Diffusion: $D + \frac{\delta_{x^2+y^2}^2}{2nt_{lag}}$	
delta (nm)	D ($\mu m^2/s$)	δ (nm)
56 ± 5	0.87 ± 0.01	37.1 ± 0.6

Confined Diffusion $D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta_{x^2+y^2}^2}{2nt_{lag}}$			
D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
0.86 ± 0.02	78105 ± 10^7	$28404 \pm 2 * 10^6$	37.1 ± 0.7

Hop Diffusion: $D_M + D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta_{x^2+y^2}^2}{2nt_{lag}}$				
D_M ($\mu m^2/s$)	D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
0.86 ± 0.02	$4 * 10^{-6} \pm 0.7$	0.7 ± 102549	0.2 ± 18129	37 ± 6

Table 4.8: Fitting parameters for the average $MSD_{app}(t)/4t$ curve of Flavidin-tagged phospholipids to the different diffusion models (Fig.4.10).

Immobile $\frac{\delta_{x^2+y^2}^2}{2nt_{lag}}$	Free Diffusion: $D + \frac{\delta_{x^2+y^2}^2}{2nt_{lag}}$	
delta (nm)	D ($\mu m^2/s$)	δ (nm)
55 ± 5	0.81 ± 0.004	37.4 ± 0.2

Confined Diffusion $D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta_{x^2+y^2}^2}{2nt_{lag}}$			
D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
0.81 ± 0.007	29413 ± 608179	16903 ± 174761	37.4 ± 0.3

Hop Diffusion: $D_M + D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta_{x^2+y^2}^2}{2nt_{lag}}$				
D_M ($\mu m^2/s$)	D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
0.81 ± 0.01	0.01 ± 0.03	3 ± 24	10 ± 16	37.2 ± 0.6

4.1.3 The most likely diffusion mode for Flavidin- and Streptavidin-tagged lipids

As seen in the previous section, the most likely model to describe the diffusion of the protein-tagged phospholipids is the free diffusion model for both tags. In top section of Fig. 4.11, 4.12, 4.13, 4.14, we present the detailed fits of this model to the data. Compared to the case of 40nm gold-tagged phospholipids, the original trajectories are distributed more symmetrically around the average value, and there are no outliers. The ensemble average is, therefore, well suited to describe the diffusion of the protein-tagged lipids in this situation, and so is the fit of the free diffusion model.

The appropriateness of this model is even more noticeable in the localization error-subtracted data, which are found in the bottom part of Fig. 4.11, 4.12, 4.13, 4.14. The detail of this operation are explained in Section 2.2.3 for the theoretical grounding, and in Section 3.2.4 for the practical operation. The subtraction of the localization error as estimated by the model fitting in the previous section gives out a constant line, in all the fits. As it can be expected, the slowest original curves tend to negative values of D after this operation, while the faster ones tend to higher values. This is a reasonable behaviour, given that faster particles will be more difficult to visualize by our detectors, and therefore have a higher localization error. Conversely, the localization error of the ensemble average will overestimate the localization error for the slowest curves. Apart from this detail, we find that the localization error subtraction gives an immediate visual way of comparing the diffusion mode of the lipids tagged with the two different protein tags.

4.1. STREPTAVIDIN- AND FLAVIDIN-TAGGED LIPIDS DIFFUSING ON A MODEL MEMBRANE

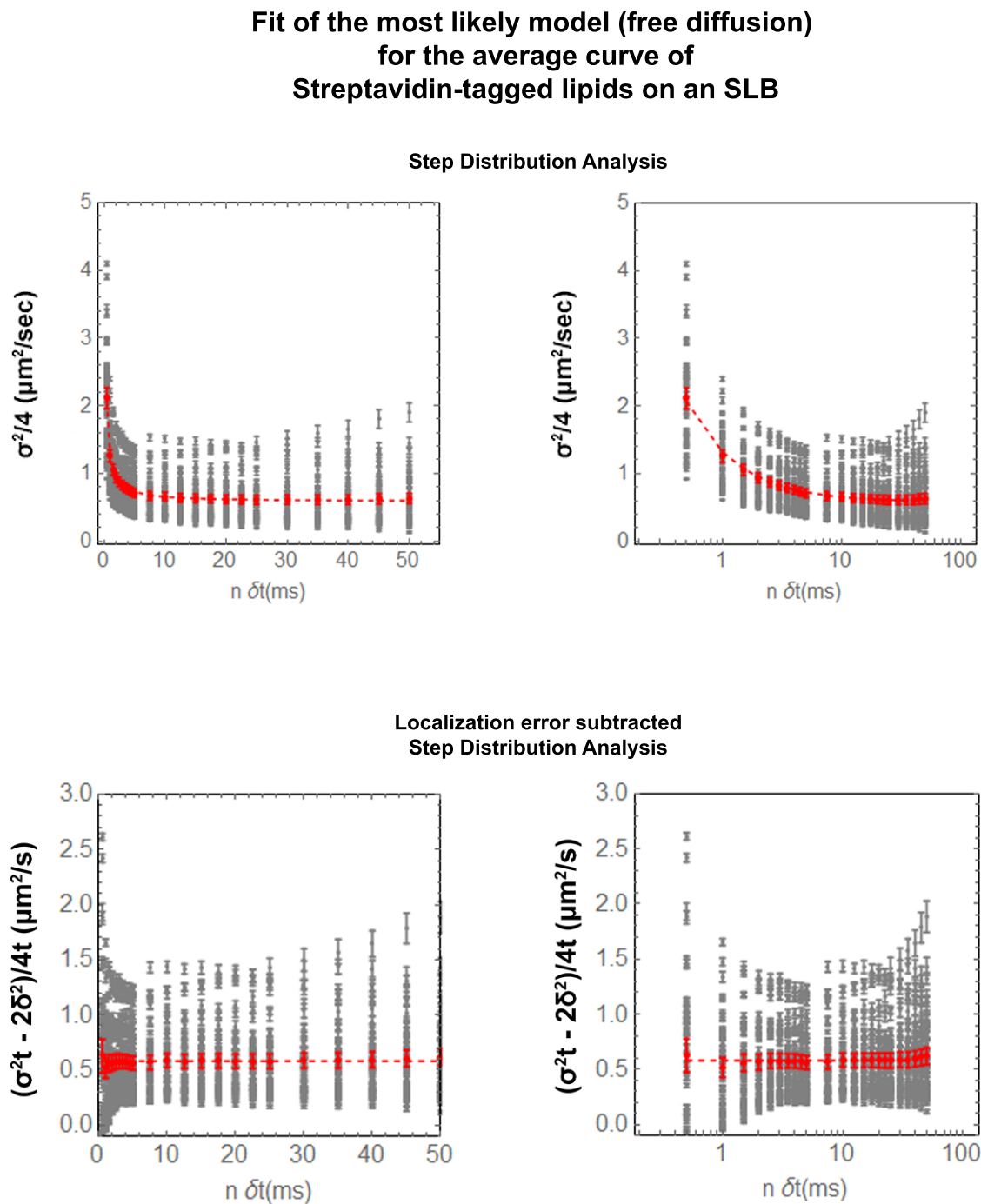


Figure 4.11: Fit of the most likely model, according to Step Distribution Analysis, for Streptavidin-tagged phospholipids diffusing on an SLB. (Top) Curve fits for the hop diffusion model, reported in linear (left) and log (right) time scale. The single trajectory data are in gray, the average $\sigma^2(t)/4$ curve is in red, and the fitted curve is in blue. (Bottom) The same data fits from the top plots, in linear (left) and log (right) time scale, where the localization error estimated through the fit has been subtracted.

**Fit of the most likely model (free diffusion)
for the average curve of
Streptavidin-tagged lipids on an SLB**

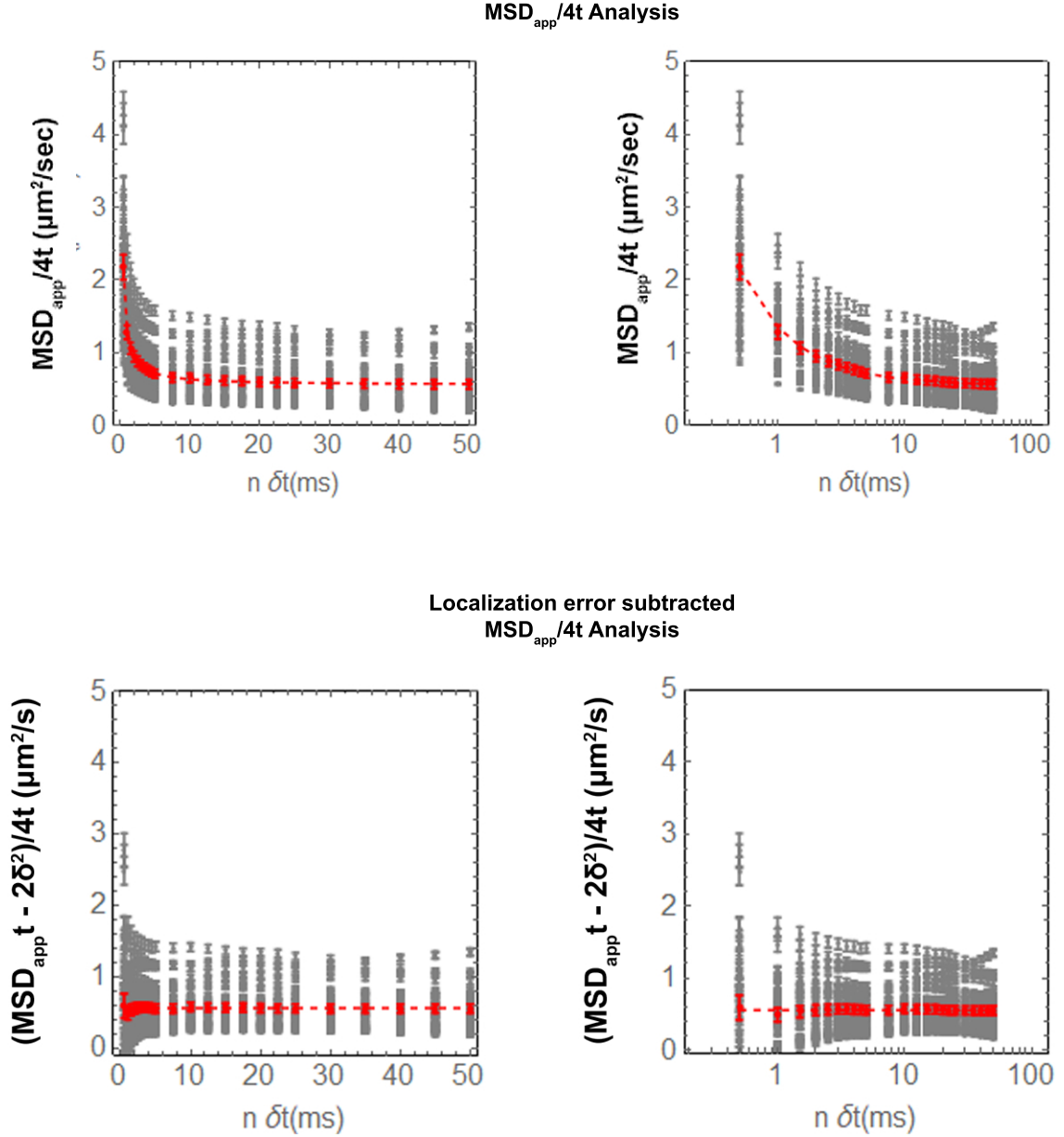
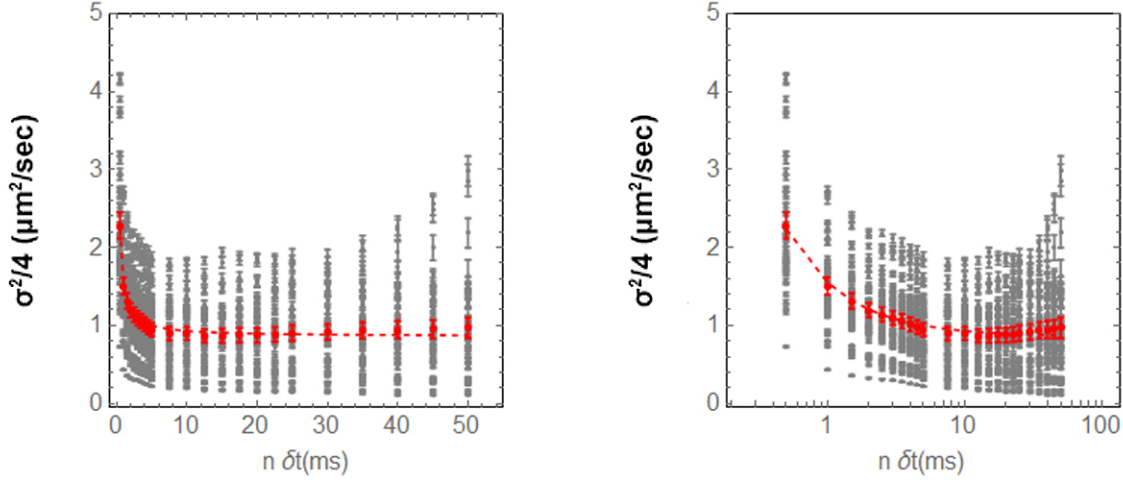


Figure 4.12: Fit of the most likely model, according to $MSD_{app}(t)/4t$ analysis, for Streptavidin-tagged phospholipids diffusing on an SLB. (Top) Curve fits for the hop diffusion model, reported in linear (left) and log (right) time scale. The single trajectory data are in gray, the average $MSD_{app}(t)/4t$ curve is in red, and the fitted curve is in blue. (Bottom) The same data fits from the top plots, in linear (left) and log (right) time scale, where the localization error estimated through the fit has been subtracted.

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Fit of the most likely model (free diffusion) for the average curve of Flavidin-tagged lipids on an SLB

Step Distribution Analysis



Localization error subtracted Step Distribution Analysis

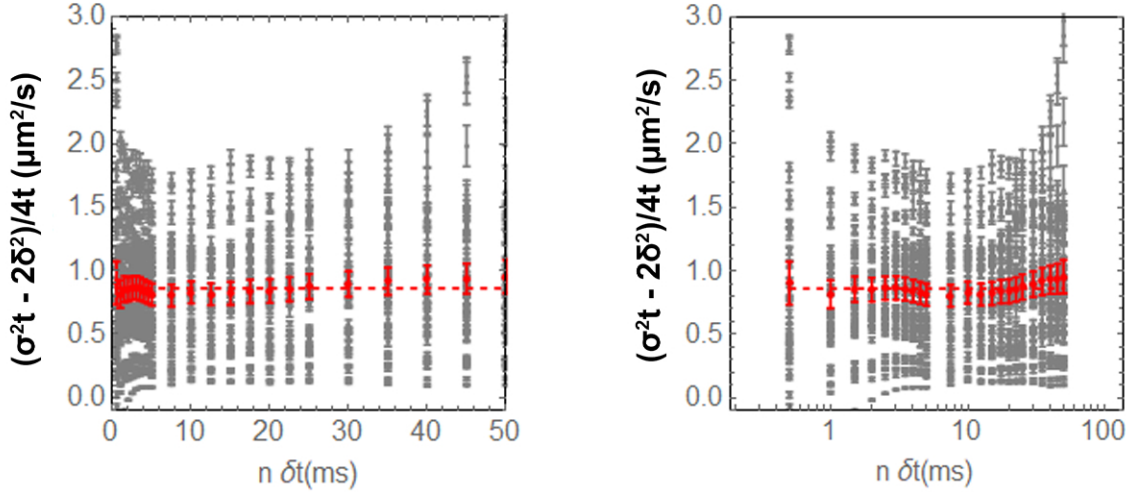
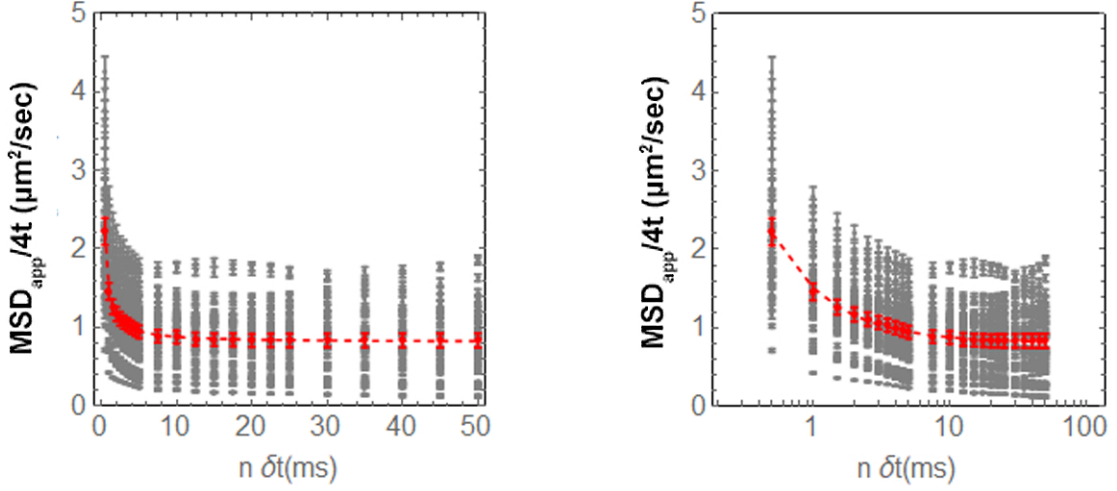


Figure 4.13: Fit of the most likely model, according to Step Distribution Analysis, for Flavidin-tagged phospholipids diffusing on an SLB. (Top) Curve fits for the hop diffusion model, reported in linear (left) and log (right) time scale. The single trajectory data are in gray, the average $\sigma^2(t)/4$ curve is in red, and the fitted curve is in blue. (Bottom) The same data fits from the top plots, in linear (left) and log (right) time scale, where the localization error estimated through the fit has been subtracted.

**Fit of the most likely model (free diffusion)
for the average curve of
Flavidin-tagged lipids on an SLB**

MSD_{app}/4t Analysis



**Localization error subtracted
MSD_{app}/4t Analysis**

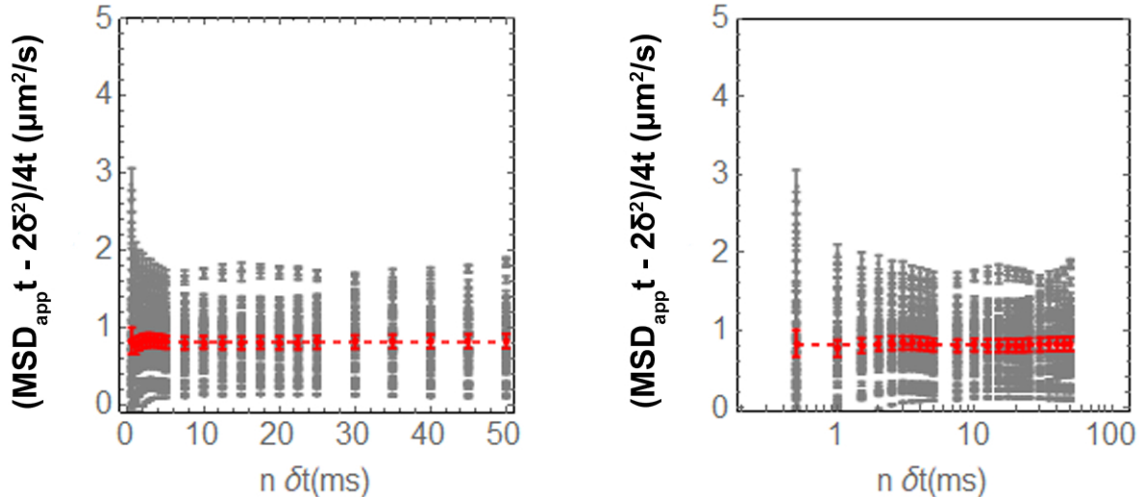


Figure 4.14: Fit of the most likely model, according to $MSD_{app}(t)/4t$ analysis, for Flavidin-tagged phospholipids diffusing on an SLB. (Top) Curve fits for the hop diffusion model, reported in linear (left) and log (right) time scale. The single trajectory data are in gray, the average $MSD_{app}(t)/4t$ curve is in red, and the fitted curve is in blue. (Bottom) The same data fits from the top plots, in linear (left) and log (right) time scale, where the localization error estimated through the fit has been subtracted.

4.2 A potential polymer tag for SPT

4.2.1 Detection and tracking

In order to confirm the results of the SPT experiments on Flavidin and Streptavidin, FCS measurements were planned on the same samples to offer a direct comparison between the two techniques. The diffusion rate of protein-tagged biotinylated lipids were compared to the diffusion of a fluorescent phospholipid analogue, DSPE-PEG(2000)-KK114. To this end, we also produced SLBs with 0.01 mol% concentration of the red fluorescent phospholipid, in a mixture of 74.9 mol% DOPC, 25 mol% Cholesterol. The visualization of such a sample with the iSCAT microscope lead to finding some diffusing particles that could clearly be observed moving on the bilayer surface. This result was surprising, since 2 kDa PEG polymers should not be able to scatter enough to be detected while diffusing. In order to test whether the detected particles were not due to impurities, we produced a sample with DOPE-cap-Biotin, a biotinylated phospholipid with a smaller linker. The comparison between these two samples is given in Fig. 4.15. In the DOPE-cap-Biotin enriched bilayer, no particle could be observed, whereas any repetition of the SLB with DSPE-PEG(2000)-KK114 or DSPE-PEG(2000)-Biotin clearly contained moving features.

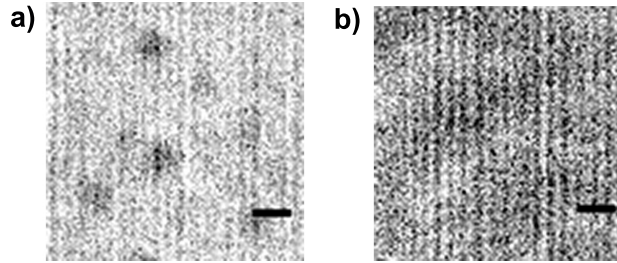


Figure 4.15: Comparison between an average-filtered iSCAT movie frame recorded for a DSPE-PEG(2000)-KK114-enriched SLB (a), and a DOPE-cap-Biotin-enriched one (b), recorded at 2 kHz sampling rate. The DSPE-PEG(2000)-KK114 doped SLB presents some low SNR particles which can be seen diffusing. These are completely absent in the SLB with DOPE-cap-Biotin bilayer. Scale bars (black) = 500 nm.

While the detected moving fraction have a lower Signal-to-Noise Ratio than the aforementioned protein tags, they can nevertheless be detected and tracked by the software employed. Therefore, the decision was taken to track them anyway, as these data could potentially unlock the possibility of using even smaller scattering tags for SPT experiments, which could be a desirable finding. The detected tracks are collected, and shown, in Fig. 4.16. The sampling rate (2 kHz) and illumination

conditions (7.3 mW laser power at $\lambda = 650$ nm) were kept consistent with the protein-tagging experiments.

The tracks were then analyzed following the same analysis method employed for the Flavinin and Streptavidin tags. The classification of the tracks based on the three diffusion models (free, confined, hop) is presented in Fig.4.17. Notably, the two analysis methods seem to disagree in the analysis of this particular sample. In fact, while the Step Distribution $\sigma^2/4$ curves seem to be described, for the best part, by the Free diffusion model, a clear majority does not emerge for the $MSD_{app}(t)/4t$ analysis. The issue is not improved with the more stringent “Fit Quality” condition applied. None of the two analysis method is necessarily more exact than the other. We shall therefore continue with the analysis of the ensemble average curves to give an interpretation to these results.

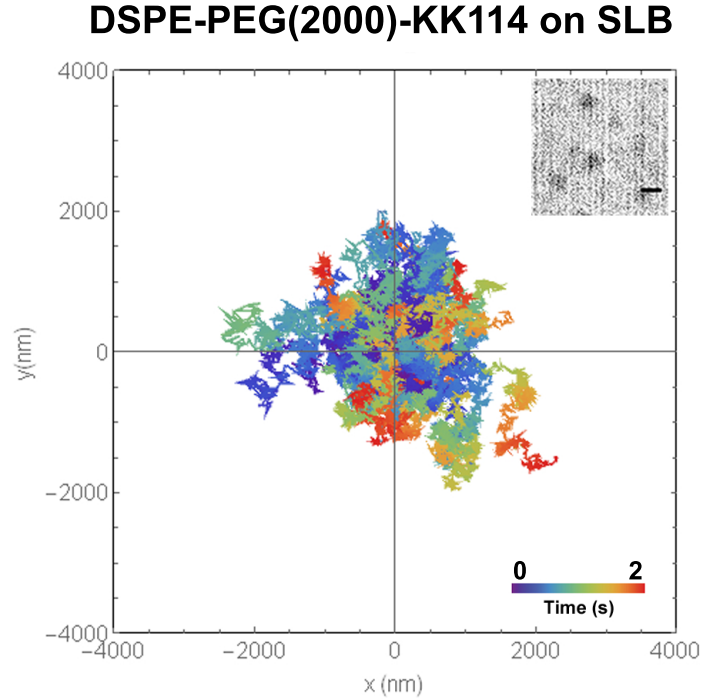


Figure 4.16: Detected tracks for DSPE-PEG(2000)-KK114 diffusing on a SLB, recorded at 2 kHz. The tracks are remapped to start at (0;0) coordinate, and go from blue ($t=0$) to red ($t=2$ s). The minimum track length is 500 positions. The number of tracks analyzed is $N = 45$. In the insert, a movie frame of the particles as detected by the iSCAT microscope. Scale bar (black) = 500 nm.

DSPE-PEG(2000)-KK114 on SLB

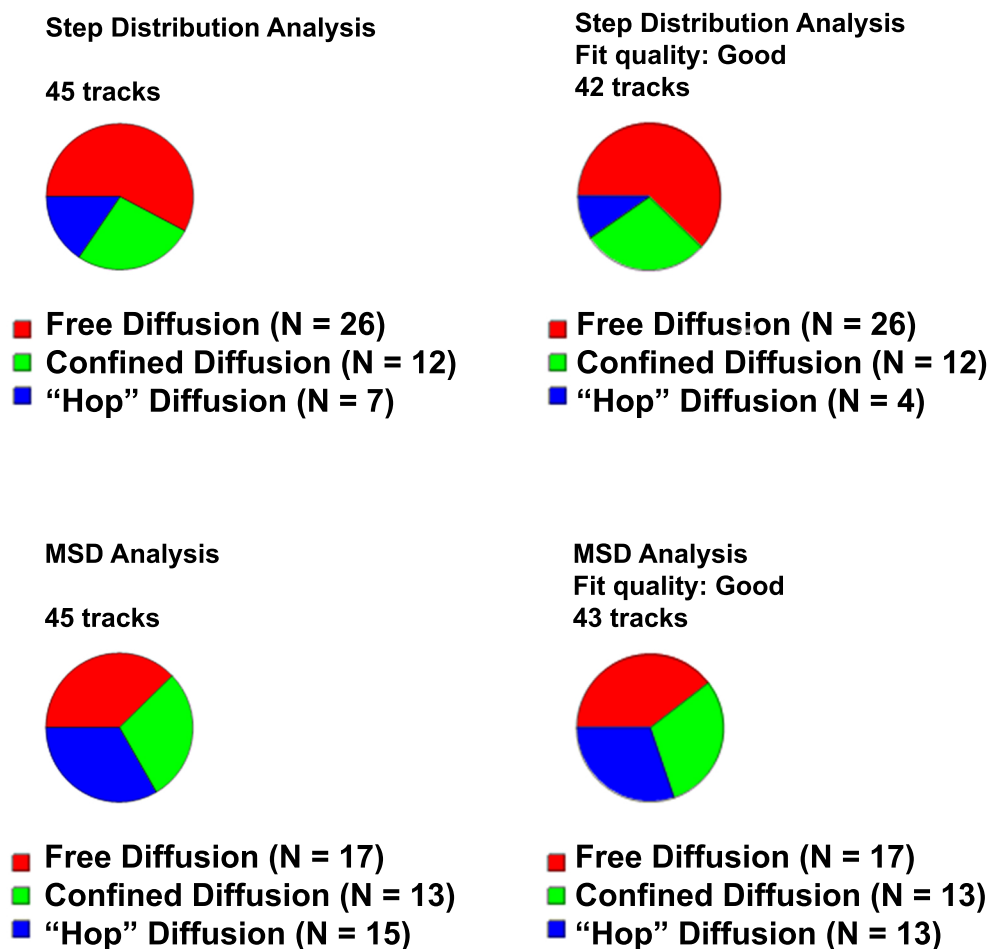


Figure 4.17: Pie charts summarizing the diffusion models fitting results for the $MSD_{app}(t)/4t$ and Step Distribution analysis for the detected DSPE-PEG(2000)-KK114 lipids on an SLB. On the left: charts for the Step Distribution (top) and $MSD_{app}(t)/4t$ analysis, where the tracks have been categorized by selecting the fitting model with the highest Relative Likelihood (calculated from the BIC). On the right: the same charts as the left, with the added condition that the most likely model should also satisfy the condition, for each of the parameters, that $p < 0.34$.

4.2.2 SPT analysis for iSCAT-detected DSPE-PEG(2000)-KK114

The SPT analysis of the average curves for the tracks detected in the DSPE-PEG(2000)-KK114-enriched SLB proceeded as in the case for the protein tags and the 40nm gold nanoparticles. Briefly, the ensemble average of the DSPE-PEG-KK114 $MSD_{app}(t)/4t$ and $\sigma^2(t)/4$ curves obtained from the original tracks was calculated, and the aforementioned models fitted to the resulting data: the immobile particle mode, and three different diffusion dynamics (free, confined, hop diffusion). In order to establish which model is the most likely to describe the average behaviour of the tracked particles, the goodness-of-fit parameters are reported in Tab. 4.9, for the Step Distribution Analysis, and 4.10 for the $MSD_{app}(t)/4t$ data. A graphical comparison of the fit results, alongside a comparison of the fitted curves alongside the data calculated from the particle tracks, is shown in Fig. 4.18.

The interpretation of these results is not as straightforward as in earlier examples. First of all, let us consider the values obtained for the Step Distribution analysis $\sigma^2(t)/4$ curve fitting. In this case, the most likely model to describe the ensemble average curve appears to be the hop diffusion one, purely according to the BIC and derived Relative Likelihood. However, such model converges badly, as reported by the “Fit Quality” metric. It is therefore sensible to consider the next most probable model, in this case the Free diffusion one, which has a relative likelihood of 0.26. This model also converges better, according to the Fit Quality metric, and is therefore more reasonable to adopt it. Moreover, this agrees with the results in Fig. 4.17, which report the free diffusion model as the ones which represents the majority of particle tracks, according to the Step Distribution Analysis. On the other hand, the ensemble average $MSD_{app}(t)/4t$ curve appears to be fitted best by the confined diffusion model. This two disagreeing conclusion apparently clash quite strongly: the confined diffusion model, in fact, tends to $D = 0 \mu m^2/s$ for large times, whereas the free diffusion model tends, obviously, to a constant value for D_{app} .

A possible convergence of the two analysis methods comes from the interpretation of the fit parameters. In fact, the free diffusion model derived from the Step Distribution Analysis yields an apparent diffusion coefficient $D = 0.56 \pm 0.005 \mu m^2/s$, while the confined diffusion, deemed the most likely by the $MSD_{app}(t)/4t$ analysis has a $D_\mu = 0.57 \pm 0.006 \mu m^2/s$. However, the confinement time $\tau = 189 \pm 30$ ms calculated is much larger than the maximum time lag included in the analysis (50 ms). Therefore, it is plausible to think that, while this expression fits the data better, we are, in fact, witnessing a “disguised” free diffusion behaviour.

4.2. A POTENTIAL POLYMER TAG FOR SPT

Although the modelling differences between the two models can be explained, these results, however, remain quite perplexing. In fact, these would suggest that the same phospholipids, tagged with Flavidin, would diffuse faster than the single unlabelled (scattering-wise) species. Furthermore, although the 2kDa polymer is at least an order of magnitude lighter than the protein tags, and therefore scatters much less than the protein tags, the localization precision of these molecules is estimated at values which are very close to those found for Streptavidin and Flavidin tags. In fact, the analysis yields values for the localization uncertainty of $\delta = 31.9 \pm 0.3$ nm (Step Distribution analysis) and $\delta = 30 \pm 0.25$ nm ($MSD_{app}(t)/4t$). A possible explanation of this phenomenon could be that the PEGylated lipids actually diffuse much faster, but since they are lighter than other tags, our tracking algorithm is biased towards detecting the slower molecules. Another way of interpreting this is that some of the diffusing lipids form clusters. These structures could originate from non-specific interactions between the PEG polymers, and their larger size lead to an increase their scattering cross-section. However, we did not attempt to find a definitive answer to the observed phenomenon.

Table 4.9: Goodness-of-fit parameters for the Step Distribution Analysis data fits for DSPE-PEG(2000)-KK114 lipids diffusing on an SLB

Fit Model	Reduced χ^2	BIC	Relative Likelihood	Fit Quality
Immobile	0.6	36.5	$3 * 10^{-33}$	good
Free Diffusion	0.9994	-110	0.26	good
Confined Diffusion	0.9994	-107	0.07	bad
Hop Diffusion	0.9996	-112.7	1	bad

Table 4.10: Goodness-of-fit parameters for the $MSD_{app}(t)/4t$ data fits for DSPE-PEG(2000)-KK114 lipids diffusing on an SLB.

Fit Model	Reduced χ^2	BIC	Relative Likelihood	Fit Quality
Immobile	0.59	35.05	$3 * 10^{-35}$	good
Free Diffusion	0.999	-102	$2 * 10^{-5}$	good
Confined Diffusion	0.9997	-124	1	good
Hop Diffusion	0.9997	-121	0.2	bad

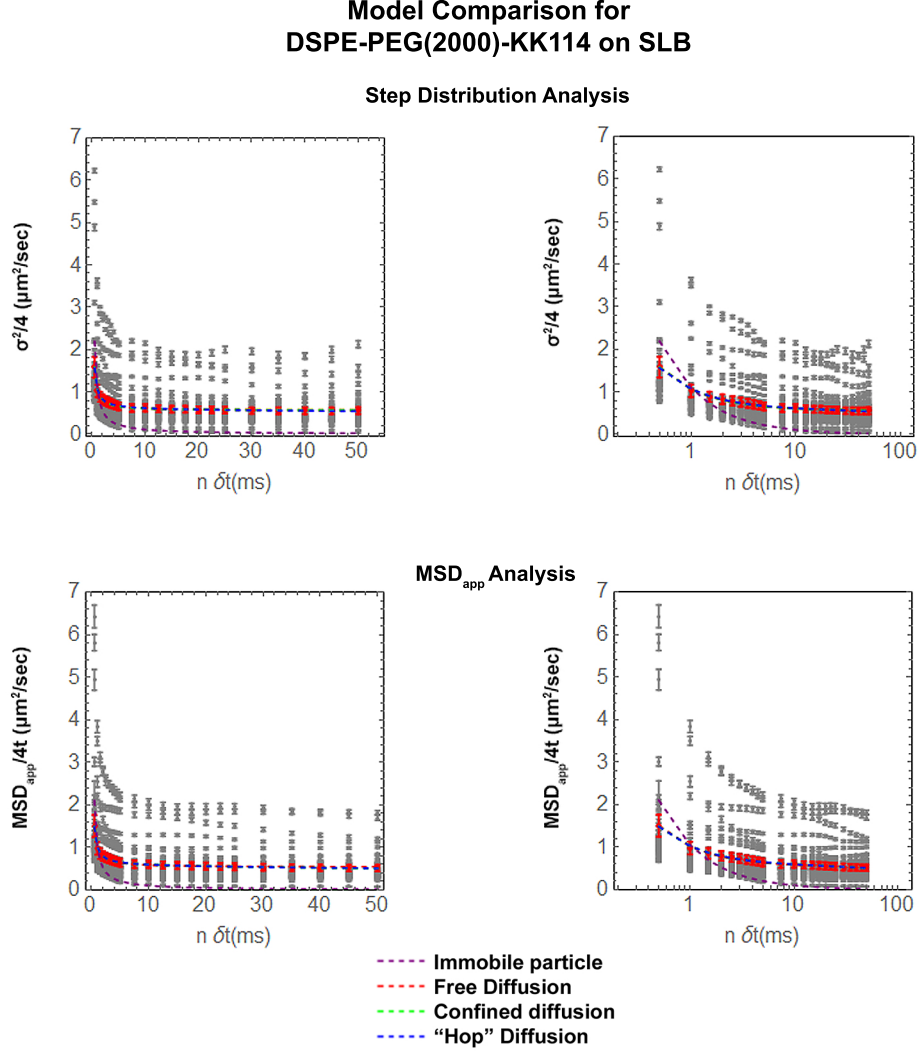


Figure 4.18: Comparison of the fitting models for the ensemble averages of the data for DSPE-PEG(2000)-KK114 lipids diffusing on an SLB.] (Top) Step Distribution Analysis: original tracks (black), average curve (red), fitted models (dashed lines, colors as in the included legend). Left: time axis in linear scale. Right: time axis in log scale, to accentuate on the first timepoints. (Bottom) Same model fittings and representations, but calculated for the $MSD_{app}(t)/4t$ curves.

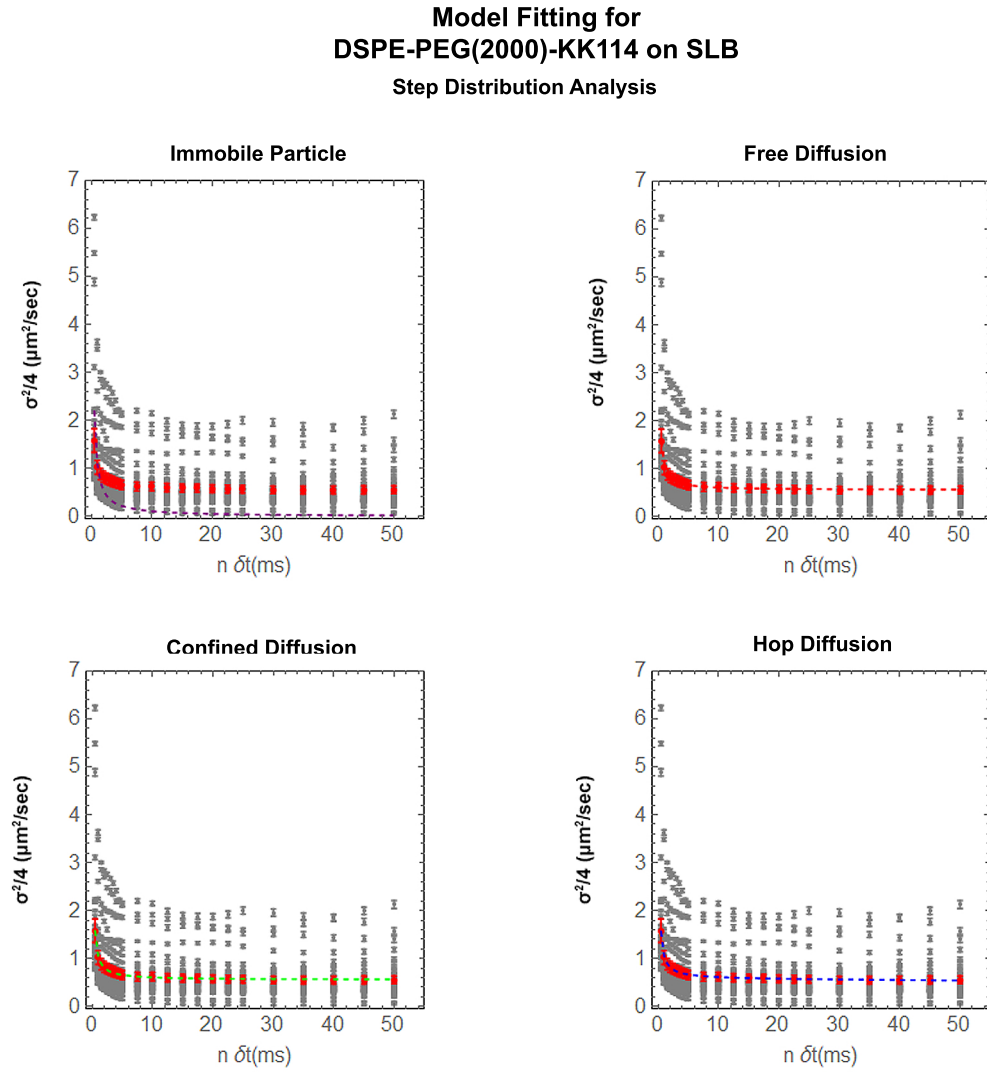


Figure 4.19: Results of the fit of the average of blur-corrected $\sigma^2(t)/4$ values extracted from the Step Distribution Analysis for all the the detected DSPE-PEG(2000)-KK114 tracks.

**Model Fitting for
DSPE-PEG(2000)-KK114 on SLB
MSD_{app} Analysis**

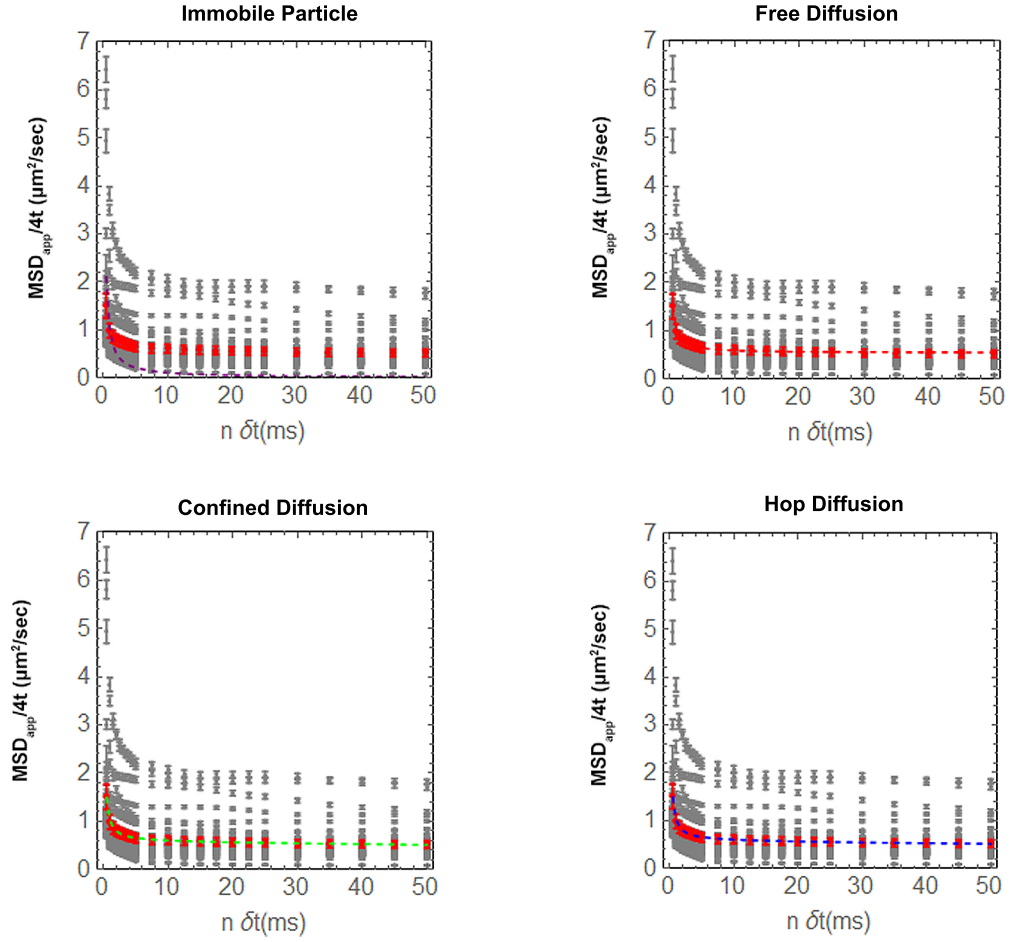


Figure 4.20: Results of the fit of the average of blur-corrected $MSD_{app}(t)/4t$ values for all the detected DSPE-PEG(2000)-KK114 tracks.

4.2. A POTENTIAL POLYMER TAG FOR SPT

Table 4.11: Fitting parameters for the average Step Distribution Analysis curve of DSPE-PEG(2000)-KK114 to the different diffusion models (Fig.4.19).

Immobile $\frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$	Free Diffusion: $D + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$	
delta (nm)	D ($\mu m^2/s$)	δ (nm)
47 ± 4	0.56 ± 0.005	31.9 ± 0.3

Confined Diffusion $D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$			
D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
0.56 ± 0.01	8547 ± 81712	7567 ± 36165	32 ± 0.3

Hop Diffusion: $D_M + D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$				
D_M ($\mu m^2/s$)	D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
0.22 ± 5	0.35 ± 5	200 ± 2991	30 ± 395	31.4 ± 0.3

Table 4.12: Fitting parameters for the average $MSD_{app}(t)/4t$ curve of DSPE-PEG(2000)-KK114 to the different diffusion models (Fig.4.20).

Immobile $\frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$	Free Diffusion: $D + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$	
delta (nm)	D ($\mu m^2/s$)	δ (nm)
46 ± 4	0.54 ± 0.005	31 ± 0.3

Confined Diffusion $D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$			
D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
0.57 ± 0.006	189 ± 30	1137 ± 93	30 ± 0.25

Hop Diffusion: $D_M + D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$				
D_M ($\mu m^2/s$)	D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
0.36 ± 0.4	0.21 ± 0.4	60 ± 142	30 ± 32	30 ± 0.3

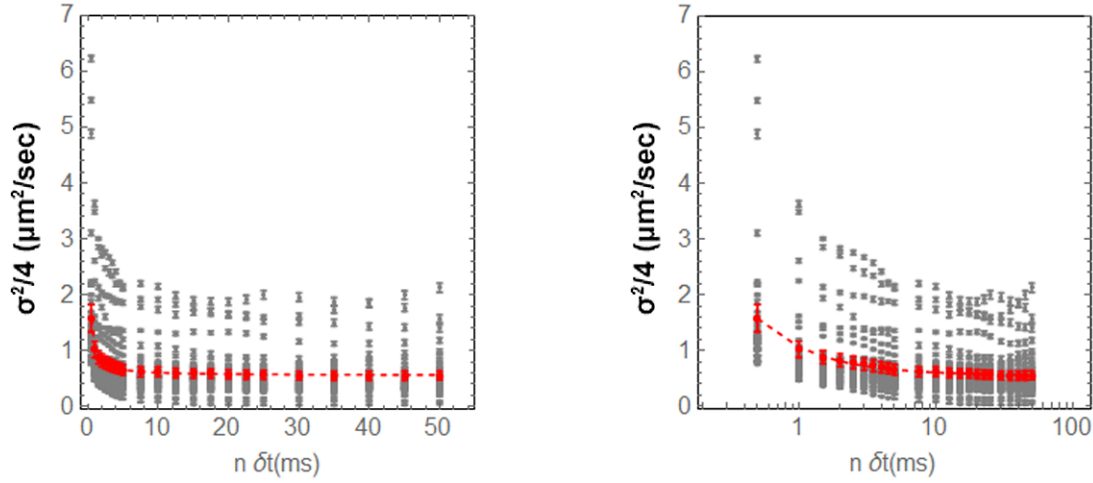
4.2.3 The most likely model for DSPE-PEG(2000)-KK114 diffusion on SLB

Despite the difficulty of interpreting the data extracted from this particular sample, we report the fits for the most likely models for both analysis methods in Fig. 4.21 and 4.22. In the top parts of this figures, we report the fit of the ensemble-averages alongside the original data. As we highlighted before, the free diffusion model is the most likely to describe accurately the ensemble average data obtained with the Step Distribution Analysis. On the other hand, the $MSD_{app}(t)/4t$ average is best described by the confined diffusion model, albeit the confinement time is, as we pointed out, much longer than reasonable. An explanation for this can be found by observing the graphs for the uncorrected data in Fig. 4.22. The average in the last points is lowered by the values of some outliers amongst the original curves, that make the ensemble average data asymptotically converge to zero. This makes the confined model more suited, at least statistically, to describe these data, although this does not reflect the actual diffusion mode of the particles. This issue could be solved by collecting more curves, thus increasing the sample size.

The analysis of the localization error-subtracted data (bottom graphs) adds new details to the considerations already made. First of all, we notice that the distribution of the original data around the ensemble average presents much faster outliers (gray curves in the bottom panels of Fig. 4.21 and 4.22). Furthermore, these outliers are characterized by a much larger localization error, as reported by the first time points. However, the majority of the curves falls around the calculated average. This could be the consequence of an implicit bias in our detection: since possibly clustered, particles diffuse slower, they will have a higher Signal-to-Noise Ratio in the final image. Therefore, they could be detected and tracked more easily. This might in turn move the average detected diffusion rates towards smaller values.

**Fit of the most likely model (free diffusion)
for the average curve of
DSPE-PEG(2000)-KK114 lipids on an SLB**

Step Distribution Analysis



**Localization error subtracted
Step Distribution Analysis**

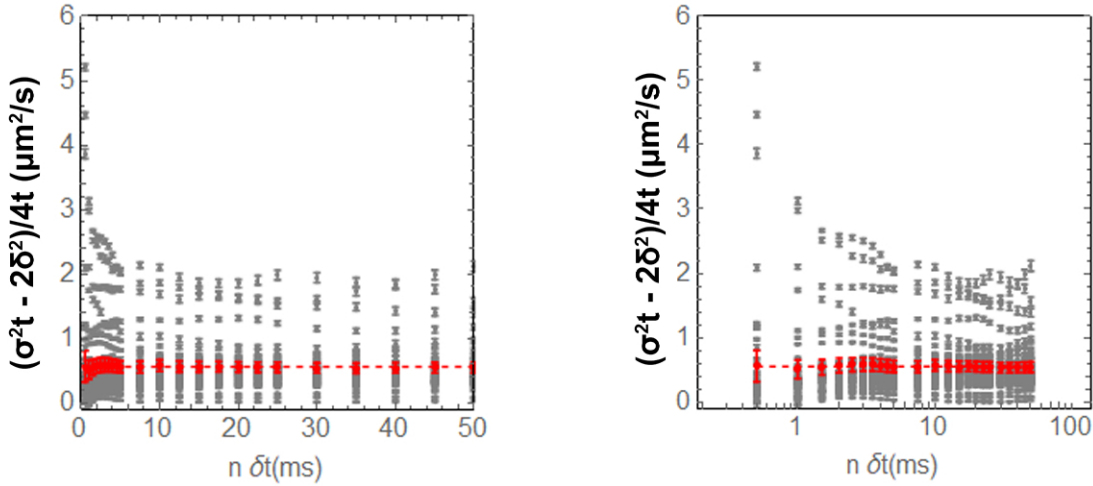


Figure 4.21: Fit of the most likely model, according to Step Distribution Analysis, for the detected DSPE-PEG(2000)-KK114 lipids on an SLB. (Top) Curve fits for the hop diffusion model, reported in linear (left) and log (right) time scale. The single trajectory data are in gray, the average $\sigma^2(t)/4$ curve is in red, and the fitted curve is in blue. (Bottom) The same data fits from the top plots, in linear (left) and log (right) time scale, where the localization error estimated through the fit has been subtracted.

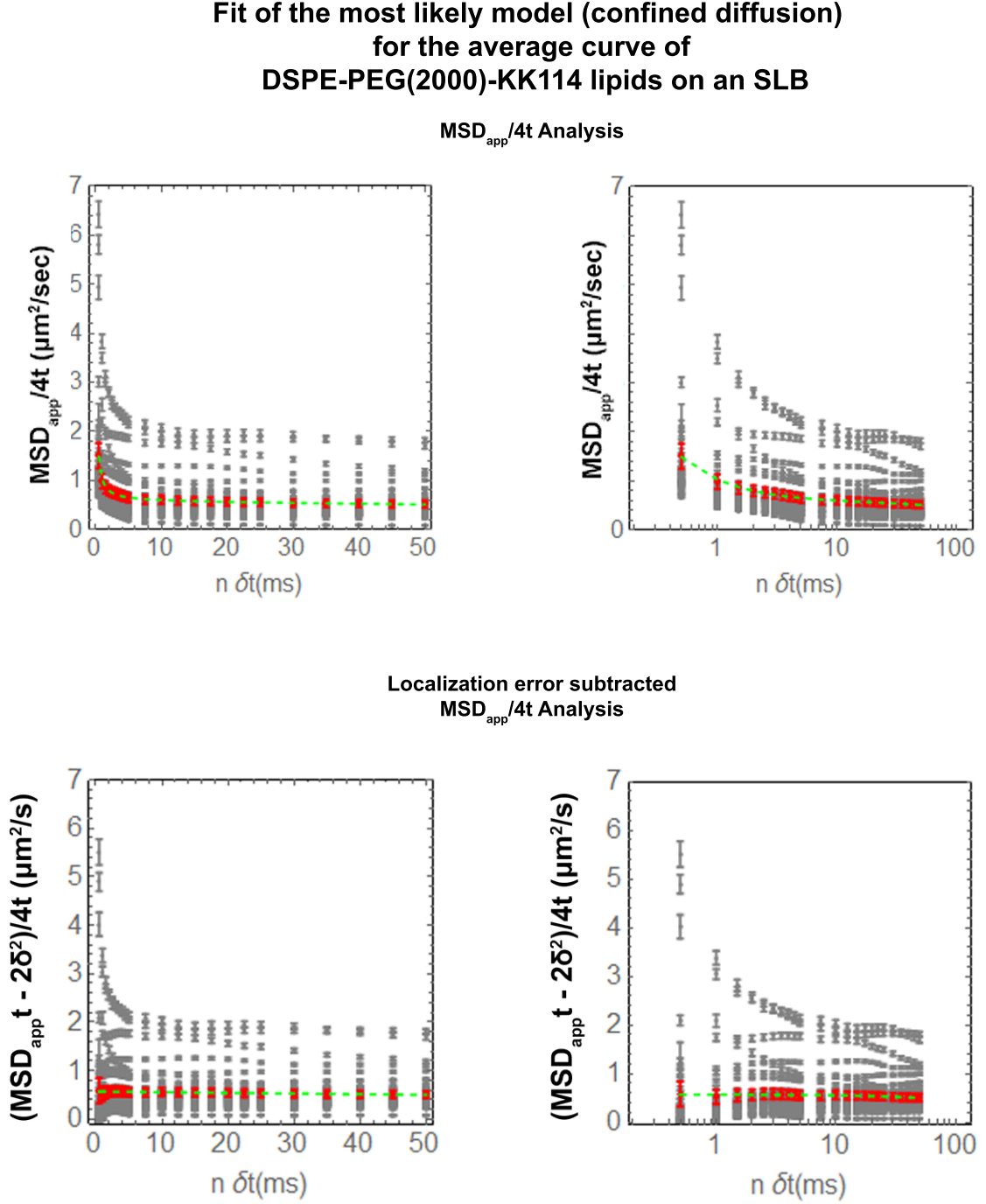


Figure 4.22: Fit of the most likely model, according to $MSD_{app}(t)/4t$ analysis, for the detected DSPE-PEG(2000)-KK114 lipids on an SLB. (Top) Curve fits for the hop diffusion model, reported in linear (left) and log (right) time scale. The single trajectory data are in gray, the average $MSD_{app}(t)/4t$ curve is in red, and the fitted curve is in blue. (Bottom) The same data fits from the top plots, in linear (left) and log (right) time scale, where the localization error estimated through the fit has been subtracted.

4.3 FCS experiments

The findings from the SPT experiments were also verified using Fluorescence Correlation Spectroscopy. The very same samples employed for the aforementioned experiments were loaded onto a Zeiss 880 LSM microscope, and the FCS autocorrelation curves were calculated by measuring the fluctuations in the fluorescence signal for 10 seconds in a variety of spots in each sample. The sample was excited using both a 633 nm laser light source, to visualize the diffusion of the protein tags and the fluorescent lipid analogue DSPE-PEG(2000)-KK114. We also collected the FCS curves by exciting the sample with a 488 nm laser light source, to simultaneously measure the diffusion of DPPE-Atto488, present in the same SLB, as a reference. Another protein tag, Neutravidin-635P (Abberior GmbH) was also measured using FCS, in addition to the ones mentioned above. This variant has a lower surface charge compared to wild type Streptavidin, and was added to these measurements to verify whether any effect was imputable to electrostatic interactions between the protein and the lipid membrane. Normalized average correlation curves are shown in Fig.4.23.

The curve in Fig.4.23 for DSPE-PEG(2000)-KK114, compared to DPPE-Atto488 presents a small variation. This is due to the fact that the observation spot size for FCS is larger when using the red wavelength excitation, necessary for the KK114 dye, compared to the blue wavelength used for Atto488 dye. This is due to the dependence of the observation spot size to the excitation wavelength, following the relation $d = \lambda_{exc}/(2N.A.)$, in which N.A. is the numerical aperture of the objective used (1.2). The curve for lipid-bound Flavidin, however, is still very similar to the one obtained for DSPE-PEG(2000)-KK114, suggesting that the difference between the two is not very significant. On the other hand, biotin-tetravalent Streptavidin and Neutravidin have curves that suggest longer correlation times. This is a phenomenon that could be expected, from what already expressed in Chapter 3 for gold nanoparticle tags. In order to compare the results, the single autocorrelation curves for all samples were fitted to a single-diffusing species model (Eq. 2.22), and the average transit times τ_{diff} collected. The distribution of these values is plotted in Fig. 4.24. For each sample, the curves for the red-fluorescent tag and for DPPE-Atto488 were averaged, and the values are summarized in Table 4.13. Using the above-mentioned relation between spot diameter and wavelength, we are also able to give an estimate for the diffusion coefficient of the protein-tagged lipids, and DSPE-PEG(2000)-KK114. We notice how the values of D derived from the FCS measurement are bigger by a factor

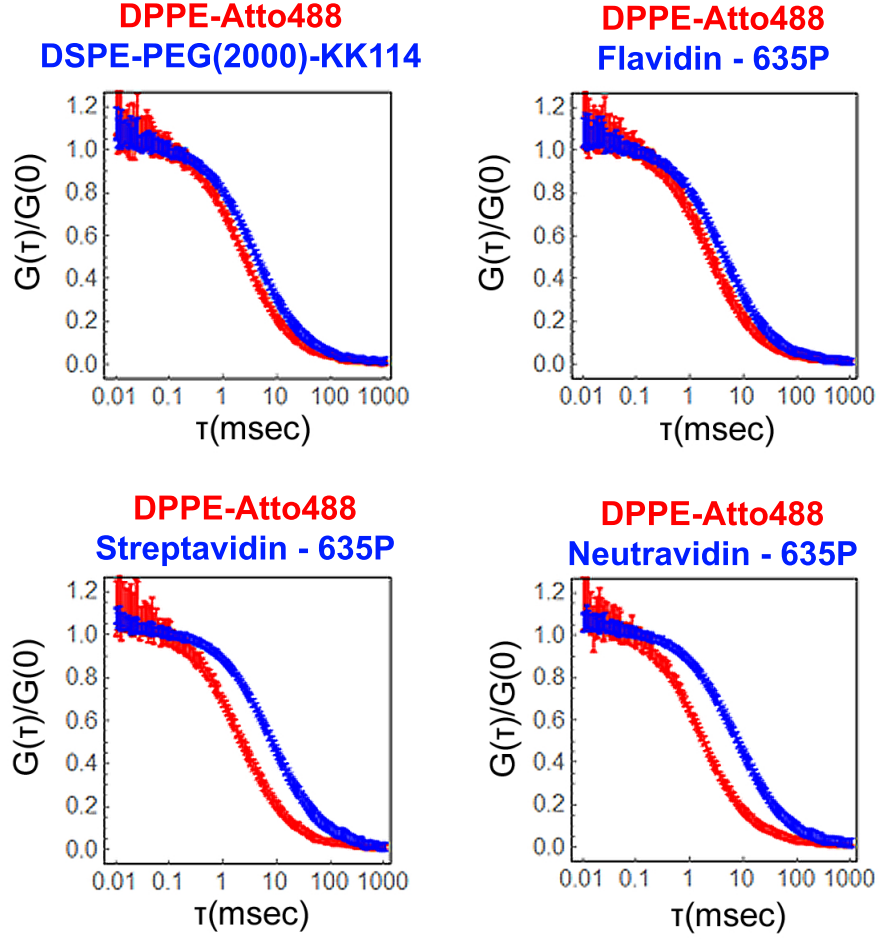


Figure 4.23: Average FCS correlation curves for the tags tracked via SPT and Neutravidin (red), compared to the same curves obtained for DPPE-Atto488 (blue). The average curves were obtained from signal fluctuations measured for a period of 10 seconds for a minimum of 11 different observations spots per sample.

of four compared to the ones obtained from the iSCAT-SPT data analysis, which is an aspect that will need investigation in future experiments.

From these values, we can see how highly variable the DPPE-Atto488 transit times are, which is probably a consequence of a very high variability in the quality of the SLBs produced by spin-coating, which is a detail that cannot be *a priori* assessed. From an accurate analysis of the τ_{diff} values, we can assert that the difference between DSPE-PEG(2000)-KK114 and Flavidin is not statistically significant ($p = 0.98$, that is, almost identical), and so is the one between Streptavidin and Neutravidin ($p=0.8$). Conversely, the difference between monovalent and polyvalent tagging is even discernible by eye as a ~ 2 fold difference in the transit times values

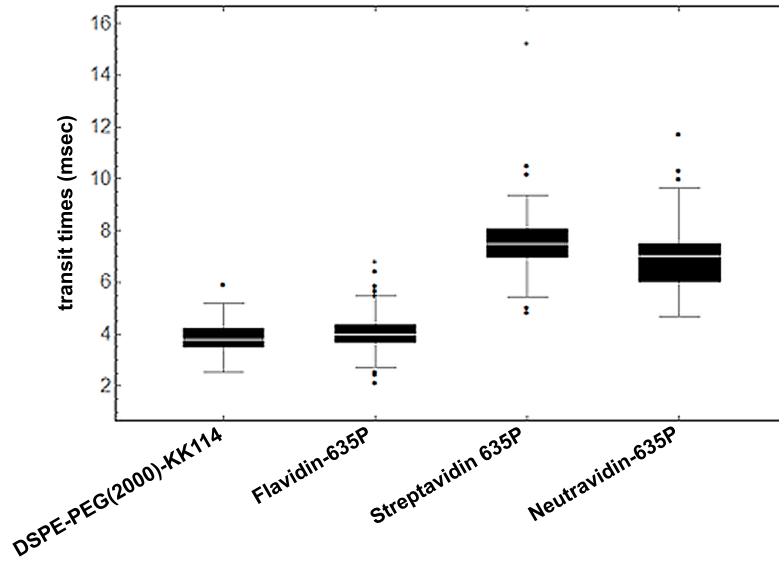


Figure 4.24: Distribution of the values for the transit times of DSPE-PEG(2000)-KK114, and DSPE-PEG(2000)-Biotin-bound fluorescent Flavidin, Streptavidin and Neutravidin.

Table 4.13: Average τ_{diff} for the red fluorescent protein and lipids and DPPE-Atto488 on SLB.

Molecule	Mean τ_{diff} (ms)	D_{app} $\mu m^2/s$
DPPE-Atto488	2.4 ± 0.4	3.1 ± 0.5
DPPE-Atto488	2.2 ± 0.6	3.4 ± 0.8
DSPE-PEG(2000)-KK114	3.9 ± 0.6	3.2 ± 0.5
DPPE-Atto488	2.1 ± 0.4	3.6 ± 0.7
DSPE-PEG(2000)-Biotin Flavidin-635P	4.1 ± 0.8	3.1 ± 0.6
DPPE-Atto488	2.1 ± 0.4	3.6 ± 0.7
DSPE-PEG(2000)-Biotin Streptavidin-635P	8 ± 1	1.6 ± 0.2
DSPE-PEG(2000)-Biotin Neutravidin-635P	7.1 ± 1	1.8 ± 0.3

($p < 0.0001$).

The only perplexing note of these measurements is that, the values of D_{app} obtained from these experiments (Table 4.13) are in the order of magnitude of a few $\mu m^2/s$. This values are ~ 4 -fold larger than the ones obtained from the analysis of SPT data of Streptavidin- and Flavidin-tagged lipids. Nevertheless, we obtained a similar difference between the diffusion rates of Streptavidin- and Flavidin-tagged lipids in FCS as in SPT.

4.4 Conclusions

In this Chapter, we have explored the characteristics of some small tags, with regards to the standards of the labels conventionally used for scattering-based detection. In fact, thanks to the outstanding properties of iSCAT microscopy, we have achieved imaging and tracking of single protein-tagged lipids, using Streptavidin and its monovalent engineered mutant, Flavidin. The importance of these two protein tags lies in the possibility of using them as a model system to investigate how tag crosslinking influences detected diffusion dynamics.

Our analysis methods proved sensitive enough to verify a difference in the diffusion rates of the lipids tagged with the two different proteins of a factor of ~ 1.6 . This was further verified by FCS experiments, which highlighted a ~ 2 -fold difference for the same quantities, even though the FCS measurements showed average transit times corresponding to larger diffusion coefficients than the ones observed in iSCAT-SPT. Therefore, from the results of both techniques we can conclude that the crosslinking potential of the tetravalent Streptavidin tag has an influence on the diffusion rate of the target molecules. We stress, however, how crosslinking to multiple lipids does not affect the diffusion mode of the labelled particles. In fact, the average observed diffusion mode is tendentially free diffusion, with only a minority of the particles exhibiting transient confinement [].

Very interestingly, we were also able to observe the diffusion of some lipids conjugated to a small (2kDa) polymer. This would be a great achievement in the field of scattering-based detection, since this kind of labelling is obviously single molecule-specific, and such a light label would have no chance of interfering, in principle, with the motion of the particle. Also, this tagging modality would have absolute single molecule specificity. However, from the results obtained from the quantitative analysis of the particle tracks, we could not derive a straightforward conclusion. In effect, since the scattering cross section of the polymer-linked lipids is very small, our detection is naturally biased towards those particles that happen to diffuse slower, or clustered ones. These moving particles would, in fact, have a higher Signal-to-Noise Ratio in the final movie, and therefore be easier to track. Nevertheless, this can lead the way into looking for even smaller tags for iSCAT detection.

Finally, the results of the FCS experiments, as already mentioned, lead to some interesting conclusions. It has been a well known fact that the biggest influence in the diffusion rate of lipids in a membrane is the drag of the membrane itself [61, 116] rather than the dimension of the label. Yet, no definitive study has been conducted

4.4. CONCLUSIONS

on isolating the influence of tag crosslinking in FCS, while this phenomenon has been already explored, and modelled, in SPT experiments on supported model membranes [64, 65]. The observations we reported both using FCS and iSCAT-SPT, show that this effect is very clearly measurable, and that should be further investigated to fully understand its effects on diffusion measurements.

Chapter 5

Tracking of Gold Nanoparticles on Plasma Membrane

Finally, in this chapter, we present the data for iSCAT Single Particle Tracking experiments on cell membranes. These experiments had been planned as the final goal of the project and indeed, at the time of planning, no other application of iSCAT microscopy to cells had been reported. The main advantages of iSCAT microscopy (affordability, tunable Signal-to-Noise Ratio, access to high sampling rates), make this technique an ideal candidate to visualize, detect, and track single molecules on cells. At the time of writing, several studies have been published in which iSCAT microscopy (and its related technique, COBRI) were applied to cells, which have already been covered in the Introduction section. Very briefly, the most notable findings of these works report the mechanism of viral infection [49], the intracellular cargo transport in cells [50], label-free visualization of proteins secreted from cells [112], and tracking the compartmentalized diffusion of GPI protein on osteosarcoma and neuronal cells [120] at 40 kHz.

The experiments hereby presented aim at uncovering the dynamics of single biotinylated, saturated phospholipids, directly tagged with 40 nm gold nanoparticles, on the surface of epithelial cells. The PtK2 cells employed were particularly favourable, as they allowed to perform tracking experiments in two dimensions. In fact, the leading edge of such cells was, in fact, observed to be particularly flat and transparent at the incident wavelengths employed. Given this case, it was possible to apply the consolidated imaging and tracking analysis protocols we have thus far employed. The acquired results lead to very interesting conclusions, somewhat in agreement with previously recorded findings in literature. These could potentially lead to a stream of experiments to give new insights in understanding lipid and, in general, molecular dynamics on the cell surface.

5.1 Detection and Tracking

The cell membrane is a very crowded environment, where many different protein and lipid species constantly diffuse. Just underneath this protective surface, cellular trafficking generates a highly dynamic and changing environment. It is therefore extremely challenging to achieve label-free SPT, or even use single protein tags, on the cell surface with a scattering-based technique such as iSCAT. Thus, we find it necessary, to restrict our measurements of lipid dynamics to the case of 40 nm streptavidin-coated gold nanoparticle-tagged lipids, which ensure a much larger Signal-to-Noise Ratio than any of the other tags thus far analyzed. With the knowledge acquired from the previous experiments, we attempted the direct tagging of biotinylated phospholipids (DSPE-PEG(2000)-Biotin) on the cell surface.

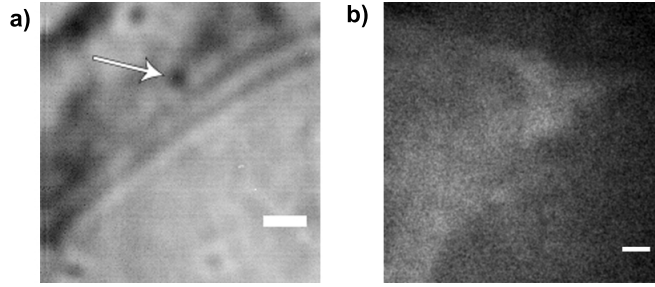


Figure 5.1: Visualization of a portion of the leading edge of a PtK2 cell in a) iSCAT Microscopy and b) TIRF. In a), we report the median-filtered image of the cell, to preserve the cell features. The arrow points to the moving particle detected on the flat edge of a PtK2 cell. In b) a contrast enhanced image of the same general area, recorded using the TIRF channel of the setup. The cells were labelled with DPPE-Atto488, a green fluorescent lipid, whose emission was excited using the 462 nm laser included in the iSCAT microscope. The particle highlighted in a) is not visible, as the gold nanoparticle is not fluorescent. Scale bars (white) = 1 μm .

The cells selected for this experiments were PtK2 cells, which is an epithelial cell line originally derived from rat kangaroo kidney tissue [149]. This cell line is one of the most studied cell lines for membrane dynamics (e.g., see [150, 66, 11, 155]), and it was readily available in Prof. Eggeling’s laboratory. The cells, seeded on 25 mm diameter glass, were kept hydrated using Leibovitz’s L15 medium (Sigma), with no phenol red. Tagging was achieved by incubating the cells at 37 °C in 500 μl of medium, where 20 μl of a solution of DSPE-PEG(2000)-Biotin was diluted to a final concentration of 0.2 mg/ml, for 30 min. The cells were also tagged with DPPE-Atto488 (4 μl of a 0.1 mg/ml solution in chloroform in 500 μl of medium) to check the appearance of the cells using the TIRF channel of the iSCAT microscope. An example of the

visualization of the cells using the two channels quasi-simultaneously is reported in Fig. 5.1. Given the characteristics of the optics employed, the iSCAT illumination had to be interrupted in order to detect the fluorescence image, to avoid spurious signal reaching the fluorescence camera.

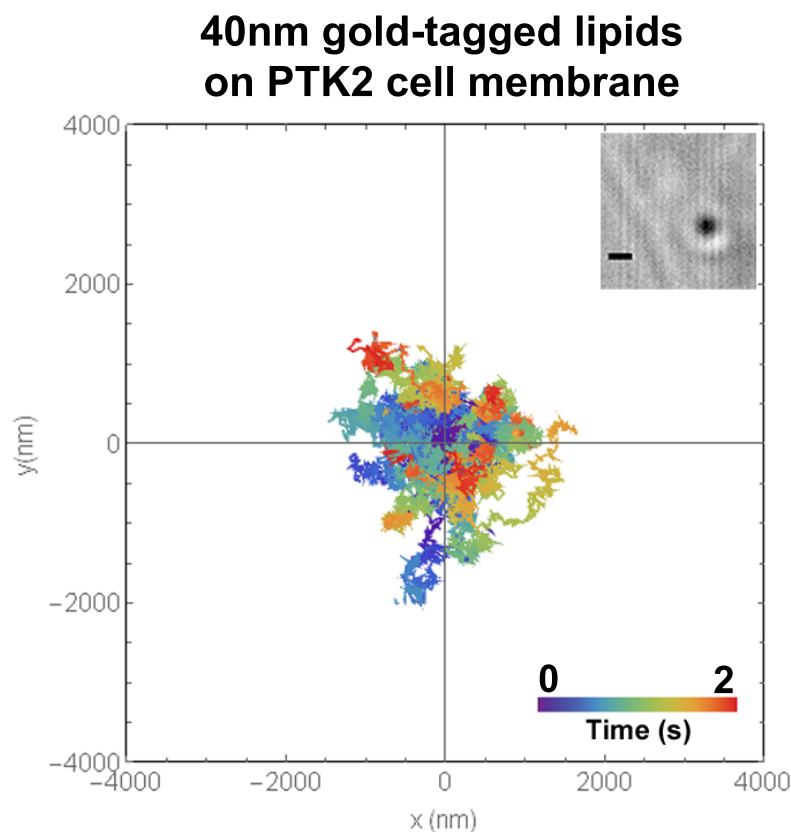


Figure 5.2: Detected tracks for 40 nm streptavidin-coated gold nanoparticles diffusing on PtK2 cell membrane, recorded at 2 kHz sampling rate. The tracks are remapped to start at (0;0) coordinate, and go from blue ($t=0$) to red ($t=2$ s). In the insert, a movie frame of one of the particles as detected by the iSCAT microscope. The analysis was restricted to the tracks longer than 500 positions, and the total number of tracks analyzed is $N=35$. Scale bar (black) = 500 nm.

The central region of the cells appeared as very crowded and heavily scattering. On the contrary, the edges were clearer, and allowed relatively easy visualization and tracking of the particles. Furthermore, the central region of PtK2 cells is much thicker than the edges. Thus the tracking in correspondence of the cell center is very challenging, due to the effect of z -coordinate change on iSCAT image generation (see also Materials and Methods, Introduction). The image quality of the TIRF microscope was deemed good enough to assess that the moving nanoparticles were indeed diffusing on the cellular membrane surface. Once this was confirmed, it was

finally possible to record the movies of the nanoparticles, and proceed to track them in the average-filtered movies. The results of the tracking operation are reported in Fig. 5.2.

As done in the previous chapters, we have analyzed the tracks by calculating the $\sigma^2(t)/4$ through the Step Distribution Analysis, and the $MSD_{app}(t)/4t$. The resulting curves were fitted to the free, confined and hop diffusion models, and classified based on the most likely model, as determined from the calculation of the Relative Likelihood based on the Bayesian Information Criterion. The results are reported in Fig. 5.3. From these charts, we can deduce that the most likely model to explain the diffusion of the gold-nanoparticle tagged phospholipid is the hop diffusion model. The $MSD_{app}(t)/4t$ analysis indeed shows that more than 75% of the analyzed tracks can be well described through this model, whereas this majority is slimmer when using the Step Distribution Analysis. This preliminary result is in agreement to those acknowledged in the literature for similar single particle tracking experiments on the same kinds of cells. Moreover, the short-lived confinements typical of this diffusion mode are often considered a proof of the compartmentalization of the plasma membrane [6, 10, 66].

40nm Gold Nanoparticles on PTK2 cell membrane

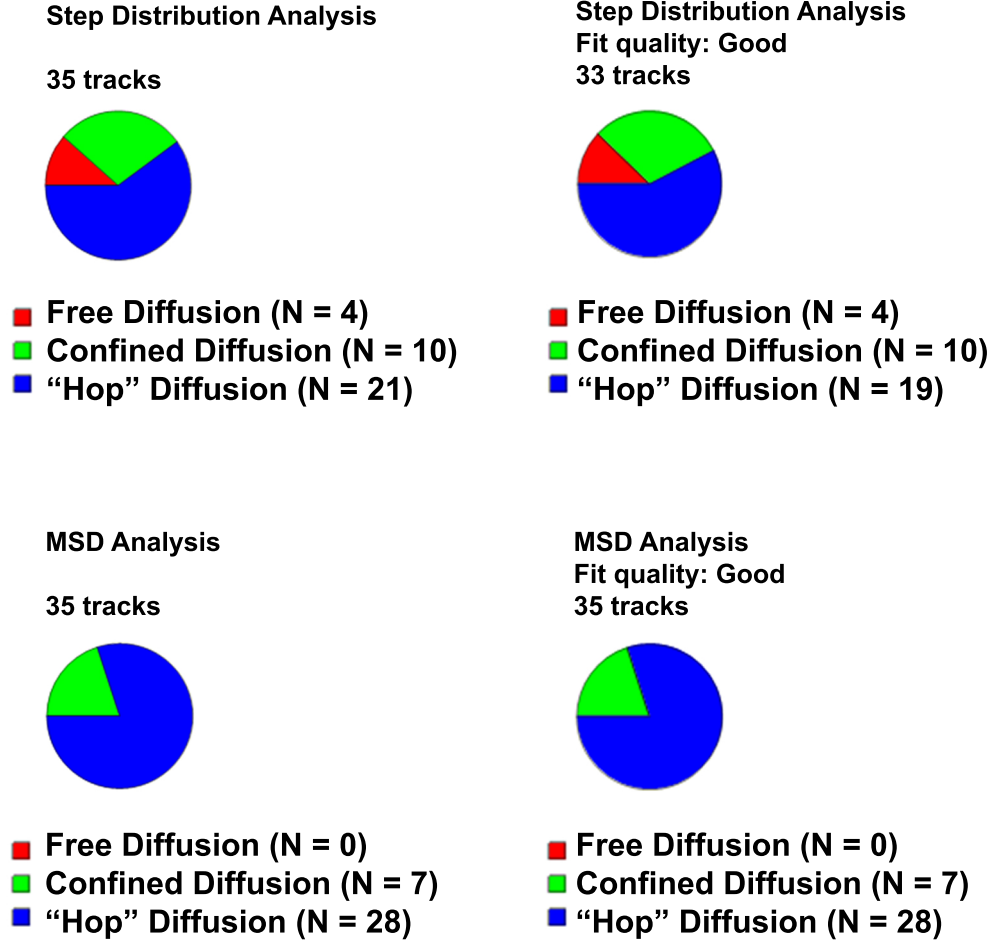


Figure 5.3: Pie charts summarizing the diffusion models fitting results for the MSD_{app} and Step Distribution analysis for 40 nm streptavidin-coated gold nanoparticle-tagged biotinylated lipids on the plasma membrane of Ptk2 cells. On the left: charts for the Step Distribution (top) and MSD_{app} analysis, where the tracks have been categorized by selecting the fitting model with the highest Relative Likelihood (calculated from the BIC). On the right: the same charts as the left, with the added condition that the most likely model should also satisfy the condition, for each of the parameters, that $p < 0.34$.

5.2 SPT analysis for the gold-tagged lipids diffusing on cells

In order to provide an idea of the most representative diffusion mode for the gold-tagged phospholipids we tracked, the ensemble averages of both the $MSD_{app}(t)/4t$ and the $\sigma^2(t)/4$ curves for all the tracked particles were calculated. As previously seen,

5.2. SPT ANALYSIS FOR THE GOLD-TAGGED LIPIDS DIFFUSING ON CELLS

we fitted four models to the SPT data: one for immobile particles, and three different diffusing particle models (free, confined, and hop diffusion). The results of the model fits are plotted in Fig. 5.4 and 5.5, and the goodness-of-fit parameters chosen are summarized in the Tables 5.1 for the Step Distribution Analysis curves, and 5.2, for the $MSD_{app}(t)/4t$ curves. The interpretation of the results is more straightforward for this sample. The “Fit Quality” metric reveals that the parameters of all the models converge well. The hop diffusion model is indubitably the best to describe the ensemble averages, as the Relative Likelihood of all the other models is negligible. We accepted this result, and move onto the interpretation of the parameters.

The fits for the single models are presented in Fig. 5.4 and Tab. 5.3 for the Step Distribution analysis average curve, and in Fig. 5.5 and Tab. 5.4 for the analysis of the average $MSD_{app}(t)/4t$ data. The D_M value, representing the “macroscopic” diffusion coefficient, that is, the diffusion between one confinement area and the other, is estimated at $0.33 \pm 0.01 \mu m^2/s$ using the Step Distribution Analysis and $0.31 \pm 0.01 \mu m^2/s$ for the $MSD_{app}(t)/4t$ curve. Therefore, the two analysis methods describe the behaviour of the particle at longer lag times in the same way. The same, unfortunately, cannot be said for the microscopic parameters. The Step Distribution analysis gives a value for the “microscopic” diffusion coefficient of $D_\mu = 0.31 \pm 0.01 \mu m^2/s$ and a confinement time $\tau = 3 \pm 0.6$ ms. On the other hand, the same parameters take the values $D_\mu = 0.43 \pm 0.02 \mu m^2/s$, with a longer confinement time $\tau = 3.7 \pm 0.5$ ms when the $MSD_{app}(t)/4t$ data are fitted. The difference between the two methods could be attributed to the presence of some positive outliers in the $MSD_{app}(t)/4t$ averaged data at shorter times, or an underestimation of these parameters by the Step Distribution Analysis.

Another interesting detail is provide by these model fits, with regards to the particle detection precision. The estimation of the localization error δ returns values of approximately 29 ± 1 nm for both analysis methods. This value is significantly different for the δ as estimated for the same 40 nm gold nanoparticles on an SLB surface (22.3 ± 0.4 nm in Step Distribution Analysis, and 19.7 ± 0.5 nm using $MSD_{app}(t)/4t$ data), and one order of magnitude lower than the ones estimated using immobilized gold nanoparticles (2.6 nm using the relative distance of two immobilized gold nanoparticles, 5nm using a single nanoparticle). This is entirely plausible, given how the cell membrane environment has a much larger scattering background compared to an homogeneous lipid bilayer, as it was for the SLB case, or a clean glass surface. However, these kind of considerations are seldom mentioned in relevant literature.

The transient confinement region on the PtK2 cell membrane that emerges from the hop diffusion model fits appears to hold the gold-tagged saturated, biotinylated DSPE lipids for few milliseconds (as witnessed by the aforementioned values of τ), in regions in the 30 to 40 nm diameter range ($L = 35 \pm 2$ nm, for the Step Distribution Analysis, $L = 40 \pm 1$ nm, for the $MSD_{app}(t)/4t$ method) (see Tab. 5.3 and 5.4). This agrees with the hypothesis that such lipids are transiently confined in subdiffraction confinement regions, the so-called “lipid rafts” [2, 3, 4], for very short time intervals. These temporary, self-forming features of the cell membrane have been particularly difficult to detect using the most commonly used microscopy techniques [12, 13]. However, we anticipate that new valuable insights could be gained to understand these phenomena by making use of the favourable experimental conditions granted by iSCAT microscopy, and its combination with SPT.

Table 5.1: Goodness-of-fit parameters for the Step Distribution Analysis data fits for 40 nm streptavidin-coated gold nanoparticle-tagged biotinylated lipids on the plasma membrane of PtK2 cells.

Fit Model	Reduced χ^2	BIC	Relative Likelihood	Fit Quality
Immobile	0.7	26.1	$6 * 10^{-31}$	good
Free Diffusion	0.986	-41	$3 * 10^{-13}$	good
Confined Diffusion	0.997	-76	$1 * 10^{-7}$	good
Hop Diffusion	0.9996	-118.7	1	good

Table 5.2: Goodness-of-fit parameters for the $MSD_{app}(t)/4t$ data fits for 40 nm streptavidin-coated gold nanoparticle-tagged biotinylated lipids on the plasma membrane of PtK2 cells.

Fit Model	Reduced χ^2	BIC	Relative Likelihood	Fit Quality
Immobile	0.7	26.1	$3 * 10^{-32}$	good
Free Diffusion	0.986	-41	$2 * 10^{-17}$	good
Confined Diffusion	0.997	-76	$7 * 10^{-10}$	good
Hop Diffusion	0.9996	-118.7	1	good

5.2. SPT ANALYSIS FOR THE GOLD-TAGGED LIPIDS DIFFUSING ON CELLS

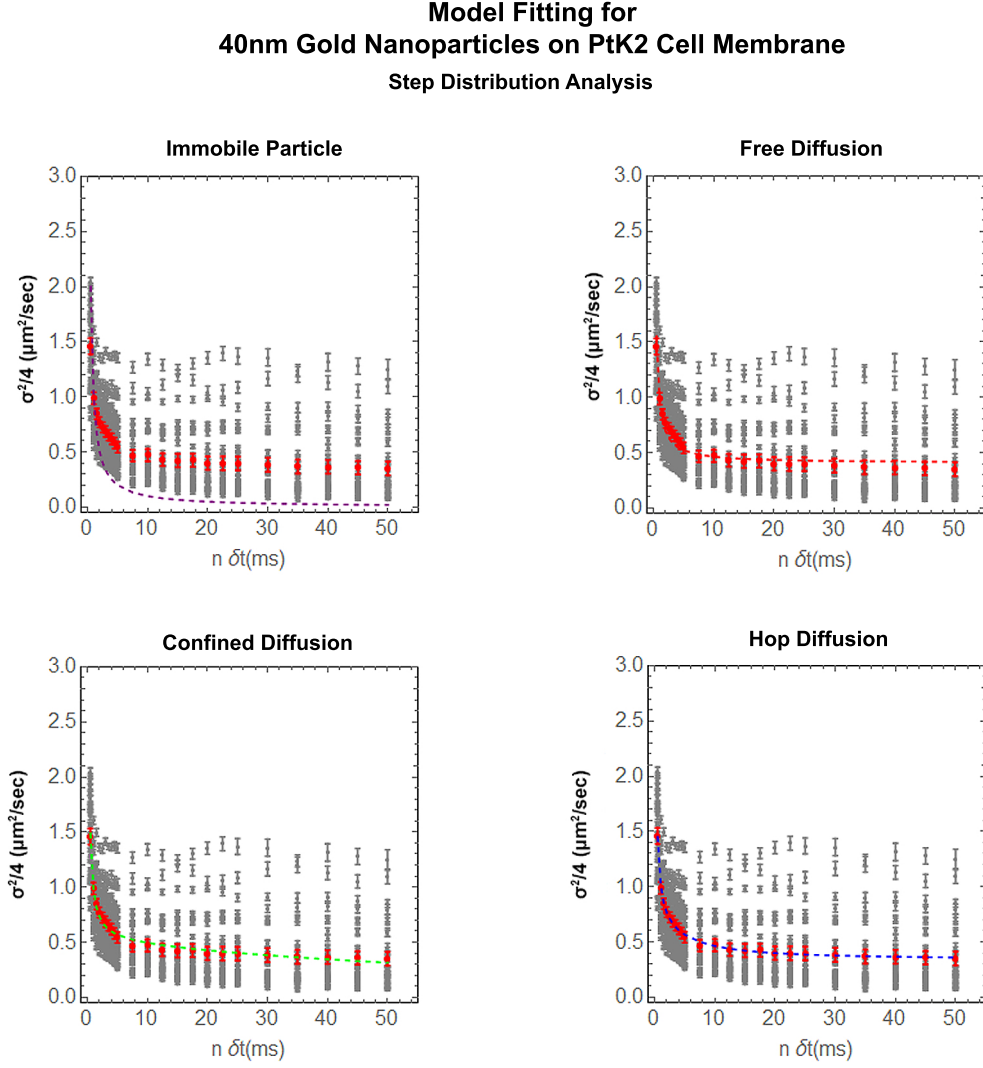


Figure 5.4: Results of the fit of the average of blur-corrected $\sigma^2(t)/4$ values extracted from the Step Distribution Analysis for all the 40 nm streptavidin-coated gold nanoparticle-tagged biotinylated lipids on the plasma membrane of PtK2 cells.

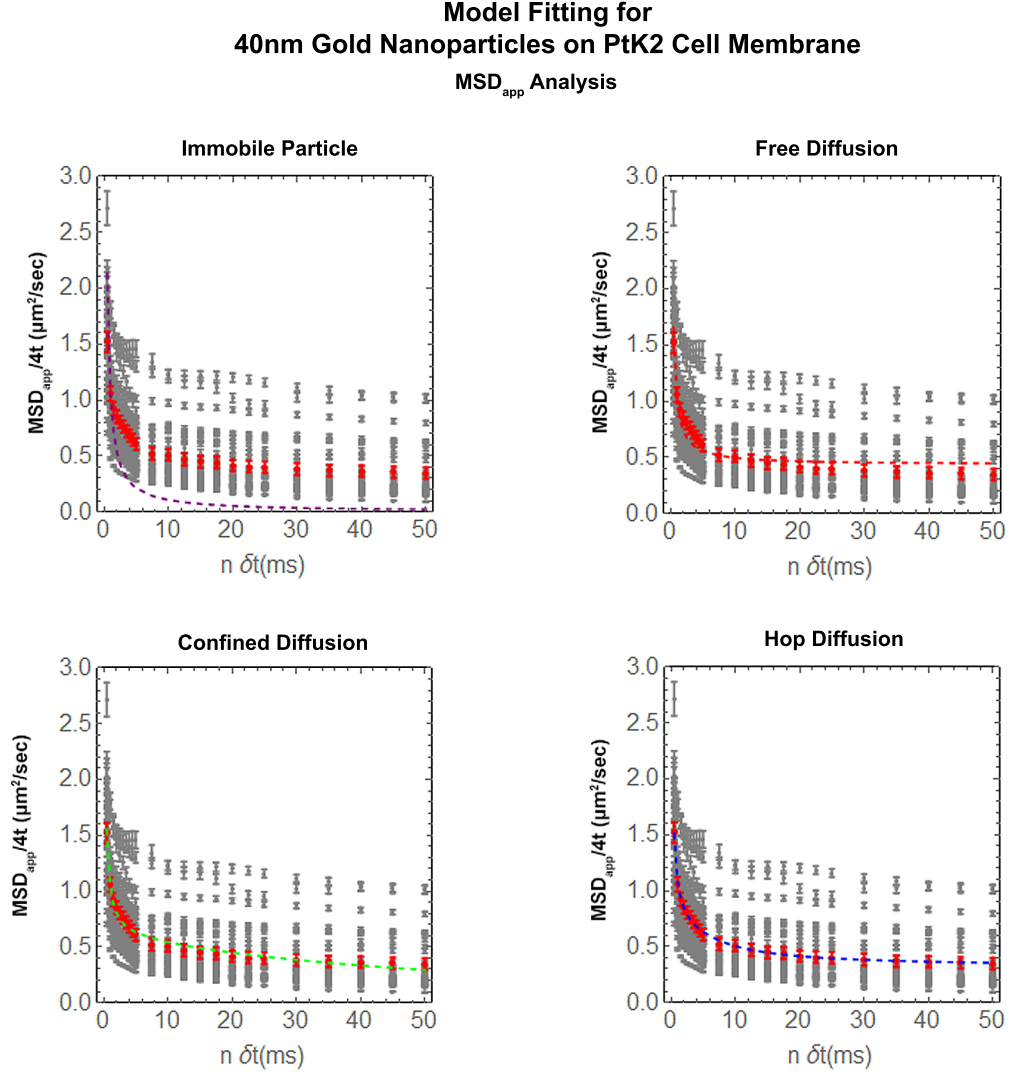


Figure 5.5: Results of the fit of the average of blur-corrected $MSD_{app}(t)/4t$ values for all the 40 nm streptavidin-coated gold nanoparticle-tagged biotinylated lipids on the plasma membrane of PtK2 cells.

5.2. SPT ANALYSIS FOR THE GOLD-TAGGED LIPIDS DIFFUSING ON CELLS

Table 5.3: Fitting parameters for the average Step Distribution Analysis curve of 40 nm streptavidin-coated gold nanoparticle-tagged biotinylated lipids diffusing on the plasma membrane of PtK2 cells to the different diffusion models (Fig.5.4).

Immobile $\frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$	Free Diffusion: $D + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$	
delta (nm)	D ($\mu m^2/s$)	δ (nm)
45 ± 3	0.41 ± 0.01	34 ± 0.8

Confined Diffusion $D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$			
D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
0.49 ± 0.02	47 ± 7	529 ± 41	32 ± 0.6

Hop Diffusion: $D_M + D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$				
D_M ($\mu m^2/s$)	D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
0.33 ± 0.01	0.31 ± 0.03	3 ± 0.6	35 ± 2	28.8 ± 0.6

Table 5.4: Fitting parameters for the average $MSD_{app}(t)/4t$ curve of 40 nm streptavidin-coated gold nanoparticle-tagged biotinylated lipids diffusing on the plasma membrane of PtK2 cells to the different diffusion models (Fig.4.8).

Immobile $\frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$	Free Diffusion: $D + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$	
delta (nm)	D ($\mu m^2/s$)	δ (nm)
46 ± 3	0.43 ± 0.02	31 ± 0.3

Confined Diffusion $D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$			
D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
0.58 ± 0.02	30 ± 4	459 ± 30	31 ± 0.7

Hop Diffusion: $D_M + D_\mu \left(\frac{\tau}{t}\right) [1 - \exp(-\frac{\tau}{t})] + \frac{\delta^2_{x^2+y^2}}{2nt_{lag}}$				
D_M ($\mu m^2/s$)	D_μ ($\mu m^2/s$)	τ (ms)	L (nm)	δ (nm)
0.31 ± 0.01	0.43 ± 0.02	3.7 ± 0.5	40 ± 1	28.2 ± 0.6

5.3 The most likely diffusion model for Gold-tagged lipids diffusing on cell membrane

Finally, we present the graphs of the most likely model to describe the diffusion of the gold-tagged lipids on the cell membrane surface in Fig.5.6 and 5.7. In the top part of these figures, we present the model fits of the hop diffusion, the most likely model for both of the analysis methods, against the ensemble average data (red), with the original data in the background. From this plots we can derive that some faster outliers are detected in this case as well, similarly to what already seen for the same gold nanoparticles on the model membrane surface. However, it still holds true in this case, as we pointed out before, that the gold nanoparticles may be crosslinking multiple lipids. Nevertheless, the confinement events here detected happen in larger confinement regions, compared to what happened in the model membrane. However, the exact nature of this confinement cannot be fully confirmed by the present experiments. The most probable causes are the crosslinking of multiple biotinylated lipids on the plasma membrane or the confinement of the target saturated phospholipids in a nanodomain (“raft”).

From the graphs of the localization error-subtracted data we can see exactly how appropriately the hop diffusion model fits the ensemble average data, in both analysis methods. The curves (dashed blue line) has, in fact, an inflection-like point around the confinement time ($\tau \approx 3$ ms for the Step Distribution Analysis, and $\tau \approx 4$ ms for the $MSD_{app}(t)/4t$ analysis), which separates the diffusion rate during the short-term confinement D_μ , from the long term diffusion coefficient D_M . We also highlight how the log scale representation should be preferred in representing this kind of results, given that the short lag time points are the most significant towards the interpretation of the diffusion dynamics, and also as they weigh more in the fitting operation.

5.3. THE MOST LIKELY DIFFUSION MODEL FOR GOLD-TAGGED LIPIDS DIFFUSING ON CELL MEMBRANE

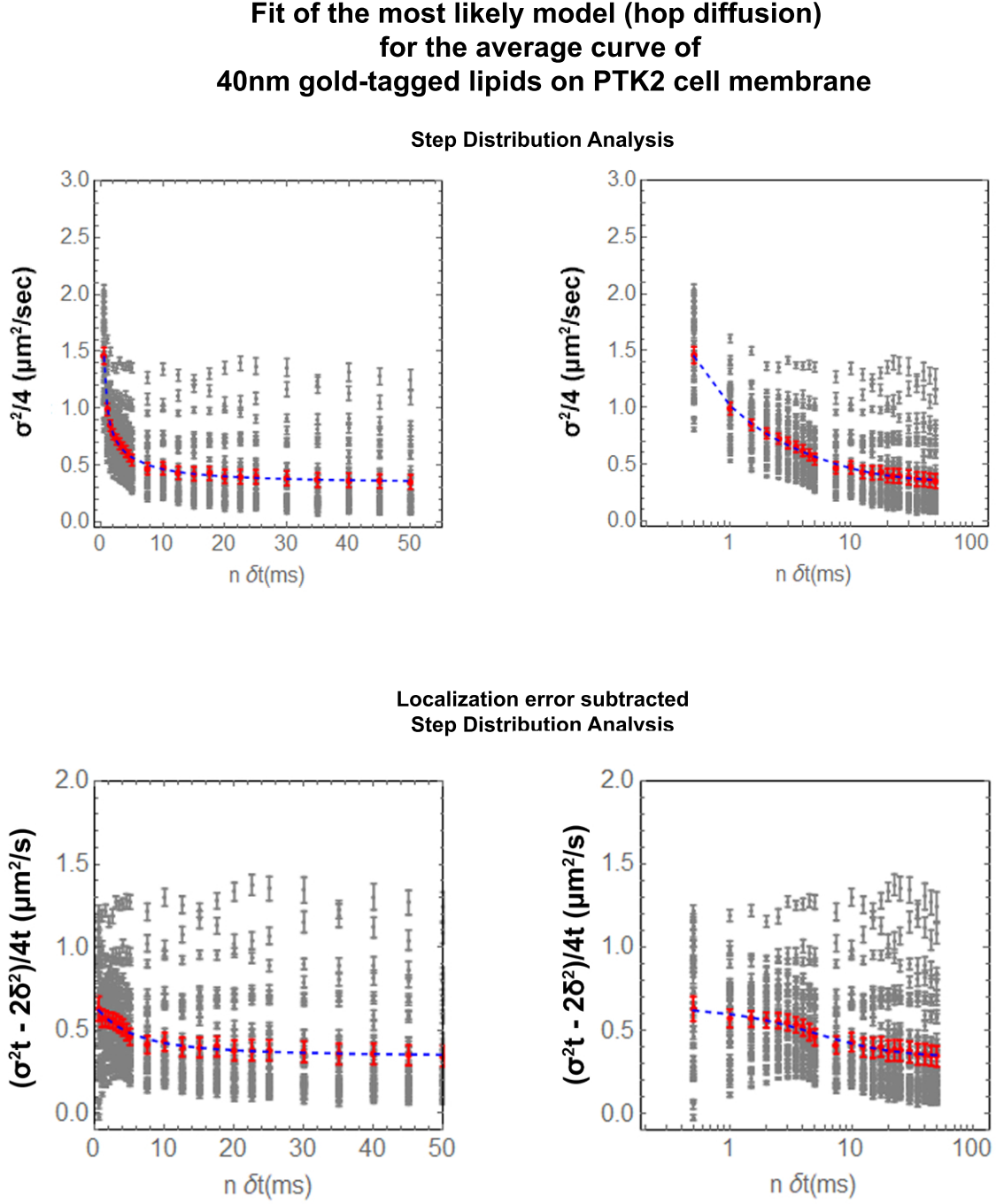


Figure 5.6: Fit of the most likely model, according to Step Distribution Analysis, for 40 nm streptavidin-coated gold nanoparticles diffusing on PtK2 cell membrane. (Top) Curve fits for the hop diffusion model, reported in linear (left) and log (right) time scale. The single trajectory data are in grey, the average $\sigma^2(t)/4$ curve is in red, and the fitted curve is in blue. (Bottom) The same data fits from the top plots, in linear (left) and log (right) time scale, where the localization error estimated through the fit has been subtracted.

**Fit of the most likely model (hop diffusion)
for the average curve of
40nm gold-tagged lipids on PTK2 cell membrane**

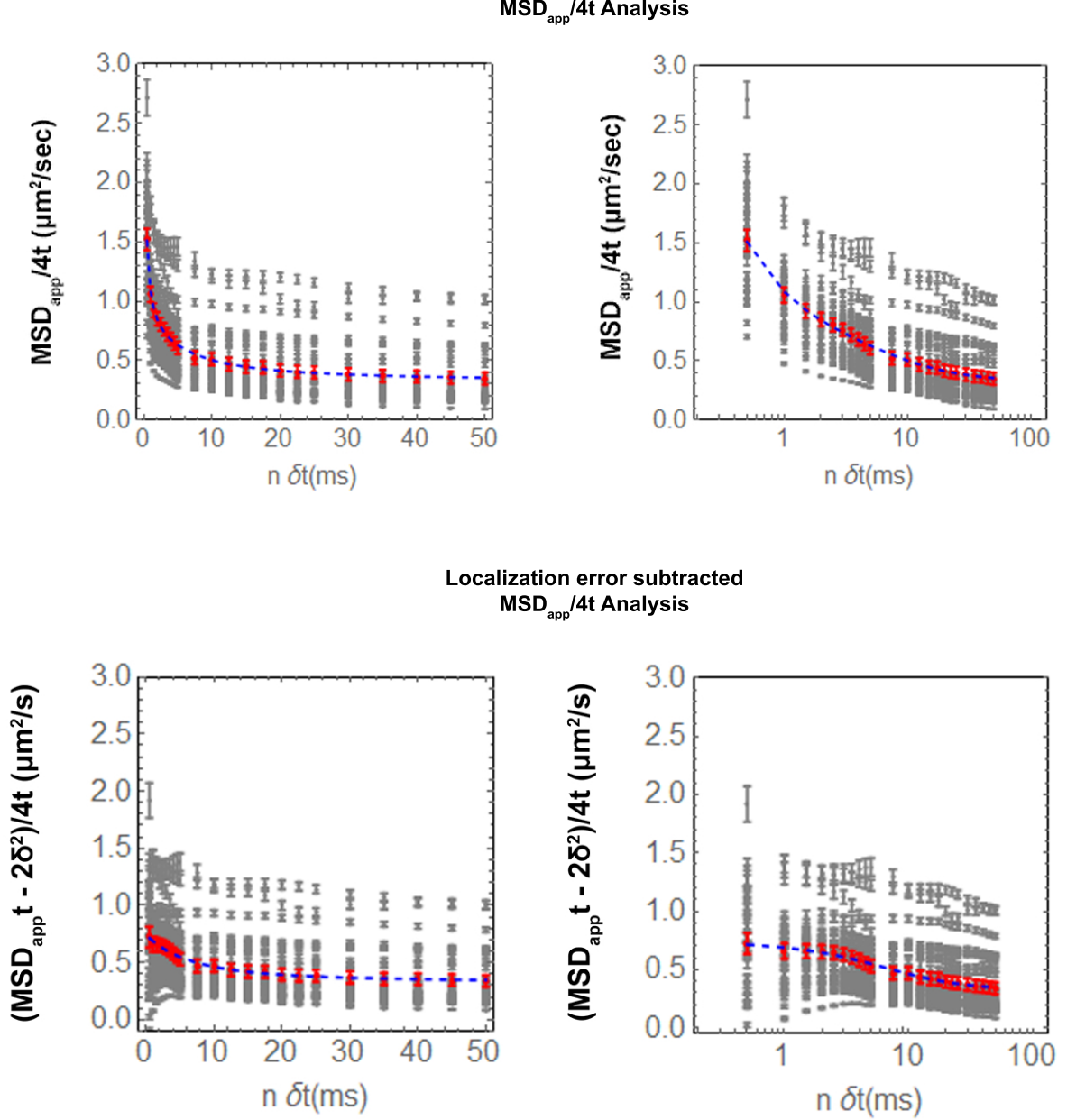


Figure 5.7: Fit of the most likely model, according to $MSD_{app}(t)/4t$ analysis, for 40 nm streptavidin-coated gold nanoparticles diffusing on PtK2 cell membrane. (Top) Curve fits for the hop diffusion model, reported in linear (left) and log (right) time scale. The single trajectory data are in grey, the average $MSD_{app}(t)/4t$ curve is in red, and the fitted curve is in blue. (Bottom) The same data fits from the top plots, in linear (left) and log (right) time scale, where the localization error estimated through the fit has been subtracted.

5.4 Conclusions

The findings reported in this chapter represent the final objective of this work, and represent a new goal in the application of iSCAT to Single Particle Tracking of the living cell. In fact, while similar experiments have already been reported in relevant literature [49, 50, 112, 120], our experiments are the first effort in tracking single lipids diffusing on the plasma membrane of epithelial cells. Also, our analysis methods introduce new details in the localization error estimation and evaluation of the most representative diffusion mode, when compared to similar experiments performed in the past [6, 10, 66].

The tracking of the saturated phospholipids tagged with 40 nm gold nanoparticles seem to confirm these well-known results: the observed lipids indeed undergo hop diffusion during the time we observed them. Furthermore, the confinements observed are short lived (τ in the milliseconds range), and happen at length scales in the tens of nanometer range. These values confirm the expectations for lipid rafts, as hypothesized in relevant literature [2, 3, 4]. However, in light of our previous findings, we cannot fully exclude just from these experiments that crosslinking could be at least concurring with these nanoscopic, transient confinements [64], since strictly monovalent tags would be required to this end. Additionally, the short-term diffusion rate we calculated through curve fitting did not exceed too much the long-term diffusion coefficient, a feature that seems to contrast with previous reports for similar SPT experiments, suggesting a much larger diffusion coefficient in confinement areas [6, 41, 43]. However, we point out that our analysis method differ substantially, especially in regards to the evaluation of the influence of localization error in the first timepoints. This difference might affect heavily the calculation of the final values for the diffusion coefficients.

Chapter 6

Conclusions

The main aim of this project was to bring the iSCAT technique closer to Life Sciences applications. The effort has been independently shared by several groups and researchers, we here presented unique contributions. One of way of achieving this was by adopting some user-friendly technical modifications to the custom built-setup. No iSCAT-specific sample preparation protocols were therefore required, and we were able to adopt similar ones as for conventional and super-resolution fluorescence microscopy. Also, a novel analysis pipeline was devised, in an effort of estimating diffusion modes and localization errors more precisely. The insights gained from the application of iSCAT to membrane diffusion are listed in this section.

First of all, our complementary STED-FCS and iSCAT investigations of the diffusion of gold nanoparticle-tagged lipids revealed a significant slow-down of the diffusion rate. Specifically, we observed a ~ 20 -fold slow-down in the diffusion coefficient of the gold-tagged lipids compared to fluorescent lipid analogues. This phenomenon was due to tag-induced crosslinking of the target molecules, and size effect. This effect appears quite obvious and intuitive when comparing nanoparticles to other kinds of labels (see Fig. 1.2), yet is often overlooked. High-sampling rate (2 kHz) experiments, further revealed that nanoparticle tag crosslinking induced transient confinements in the diffusion motion of the gold-tagged lipids. Therefore, it became apparent that fast sampling rates were necessary in order to fully appreciate such transient effects, both on model and on cell membranes.

During these observations, the need for a revised analysis pipeline became apparent. The main idea was to give a correct estimation of the effects of localization error and camera blur on the track analysis. In fact, the usually employed method of estimating the localization error, using immobilized gold nanoparticles, was found to be imprecise in our case. The observation of immobile nanoparticles, in fact, indicated the presence of external, unidentifiable sources of noise in the setup. Using

our novel analysis, we aimed at isolating and eliminating such sources of error. We therefore relied on the fitting of diffusion model to the $MSD_{app}(t)/4t$ and Step Distribution Analysis data to estimate the localization precision correctly. We highlighted how this imprecision, especially for high sampling rate experiments, heavily affects the outcomes of the analysis. Using our analysis models, the most likely diffusion model to describe the diffusion of the particles could be determined through statistical means, without prior knowledge. Yet another advantage of our analysis method was that the effects of localization errors could be estimated correctly, and even eliminated from the data plots. Thus, we could gain deeper insight on the “real” diffusion mode of the gold-tagged lipids, a step forward from the detection of the “apparent” diffusion mode which is usually observed.

Tag crosslinking was further investigated by studying the diffusion dynamics of lipids tagged with protein tags lighter than gold nanoparticles. An example of such tags is Streptavidin, a well-known biotin-tetravalent protein tag. We teamed up with Prof. Mark Howarth, who provided us with the monovalent Streptavidin mutant Mono-TrapFlavidin (Flavidin for short). This engineered protein, although having the same molecular weight (~ 50 kDa) as Streptavidin, is strictly monovalent with regards to biotin. This provided us with a simple model system to investigate tag crosslinking effects, in a way that is similar to the one presented in [64]. The Streptavidin-tagged biotinylated lipids, in fact, were found to diffuse approximately a factor of two slower than the Flavidin-tagged ones, as highlighted by complementary iSCAT SPT and FCS experiments. However, the overall diffusion mode of the target lipids was unaffected, since the most likely diffusion mode of the protein-tagged lipids was free diffusion. The results obtained from these experiments seem to fit the predictions from the numerical model developed by Camley and Brown [65], in a similar way as the experiments and simulations reported in [64].

Finally, we applied iSCAT SPT in combination with the newly-devised analysis pipeline for tracking of gold nanoparticle-tagged saturated (DSPE) lipids on the plasma membrane of epithelial PtK2 cells. We conducted our experiments at physiological temperature (37°C), and with a sampling rate of 2 kHz. The gold nanoparticle-tagged lipids showed hop diffusion behaviour. This result agrees with relevant literature [150, 66, 11, 155]. Yet, in contrast to previously reported results, we do not observe an up to ~ 10 fold increased mobility in the compartments, but rather a ~ 2 fold increase. Furthermore, given the previously acquired results on tag crosslinking and size effects, these conclusions will need further validation. This difference may

arise from our novel way of treating localization errors. As we highlight in the Introduction, in fact, the measurement errors affect more heavily the measurements at short times, where the short term diffusion is estimated. Therefore, extracting correct conclusions can be heavily challenging, and will need to be further investigated.

6.1 Future perspectives

One of the most interesting findings of this work is the possibility of detecting small polymers linked to single phospholipids, as we highlight in Chapter 4. Our analysis, however, have not been able to confirm whether the detected scattering signal came from single particles, or clusters thereof. Nevertheless, the perspective that such a small molecule could be used as a tag for scattering based detection is a very exciting prospect. More investigation in this direction could lead to isolate the right experimental conditions (e.g., incident laser power, ideal size of the polymer tag) to directly observe the diffusion of lipids with this labelling method.

According to the results we reported, the analysis pipeline developed and hereby employed showed some encouraging aspects, particularly regarding the precise estimation of localization error, and the statistical-based selection of the correct diffusion model. However, we find it necessary to validate this method in the future with ground-truth evaluations (i.e., diffusion dynamic simulations). This way, we could uniquely identify, for example, whether the Step Distribution Analysis or the $MSD_{app}(t)/4t$ method is to be preferred, making it more synthetic. The segmentation of the tracks based on the most likely diffusion mode could also be taken one step further. Instead of resorting to a complete ensemble average to have a general idea of the diffusion dynamics of the tracked molecules, it could be more appropriate to average only the tracks with the same diffusion mode, that way preserving some single-molecule detail, while at the same time attaining a reduction in the measurement error. Furthermore, the localization error subtraction could be operated on the single tracks, making it even more effective.

Finally, some further developments could also be made on the technique. At the present stage, a lot of emphasis is put on the affordability of the components, and the custom setups are still at the prototyping stage. However, the technique will surely benefit from a more user-friendly interface, both in terms of the operating hardware and the software (as already approached in a recently commercialized version of a iSCAT microscope). The combination of iSCAT with other observation methods, such as conventional Brightfield microscopy, or TIRF microscopy for Single Particle

Tracking, would be greatly beneficial for bringing the technique to a larger user base. In particular, improvements in simultaneous fluorescence observation will be necessary for further sensitizing biological experiments. Other technical improvements will be the adoption of stronger, higher quality light sources, to improve the incident light on the sample, and faster detectors, which represent the true bottleneck to the temporal resolution of the setup.

Finally, the application of iSCAT to biological studies could offer exciting new insights in single particle studies in Life Sciences, especially if further technological improvements are put in place. Refinement of the analysis method herein developed will also help to standardize Single Particle Tracking studies, and improve the shareability of results.

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