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Tracking algorithms chase down pathogens

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Abstract

Understanding sub-cellular dynamic processes governing pathogenic mechanisms is a necessary step towards the development of new drugs and strategies against infectious diseases. Sub-cellular pathogenic mechanisms, such as viral invasion processes involve highly dynamic nano-metric scale objects and rapid molecular interactions that require the study of individual particle paths. Single particle tracking methods allow to visualize and characterize the dynamics of biological objects and provide a straightforward and accurate means to understand sub-cellular processes. This review describes a number of particle tracking methods in time-lapse microscopy sequences and provides examples of using such techniques to investigate mechanisms of host-pathogen interactions.

1 Introduction

Pathogenic mechanisms are inherently governed by highly dynamic processes both at the molecular and the cellular level. Understanding and interfering with these processes are necessary steps toward the development of new drugs and strategies against infectious diseases. The path towards this goal has been significantly enlightened over the past decades thanks to the numerous advances in fluorescent probes, labeling techniques and microscopy systems, which allow one to look directly at the biological system at the appropriate scale both in space and time. Recent fluorescent imaging protocols have thus allowed scientists to shed light on a large amount of biological phenomena both at the cellular and sub-cellular level, many of which had never been observed in other imaging modalities [1].

The study of a dynamic process is intricately related to its spatio-temporal scale, both on the imaging and on the quantification side. More specifically, the distinction between cellular and molecular scales has lead to different imaging techniques and different approaches to analyze the resulting images. This review focuses on nano scales while a companion paper in a future issue will cover cellular scales.

Sub-cellular pathogenic mechanisms, such as viral invasion and spreading of prions involve highly dynamic nano-metric scale objects and fast interactions. Indeed, pathogens usually exhibit rapid motion within cells, especially when supported by an active transport mediated through F-actin, myosin or motors of the microtubule network [2]. For example, viruses can spread from cell to cell on small protrusions either driven by actin polymerization or along thin connecting fibers [3], and tunneling nanotubes may also represent a means of cell-to-cell communication used by prions for intercellular spread [4]. The characterization of these fast processes is of major interest to understand

pathogenic mechanisms. For instance, viral infection processes include many steps involving dynamic interactions with different cellular structures such as the binding of the virus to specific receptors or attachment factors on the cell surface, and the transport to specific sites mediated by the cell cytoskeleton. The diversity of these interactions, and the possible existence of alternative infection routes, makes it necessary to study individual viral particles paths. Single particle tracking allows one to visualize and characterize the path of dynamic nano-metric objects, and provides a straightforward and accurate means to understand pathogenic mechanisms. Single-virus tracking has consequently emerged over the past few years as a powerful technique for the study of virus trafficking (see [3] and references therein for a comprehensive review) or prions spreading [4].

This review describes a number of particle tracking methods in time-lapse microscopy sequences, from the earlier and generally simpler techniques up to the most recent and more evolved tracking methods. It also provides recent examples from our own work that used particle tracking algorithms to investigate infectious diseases mechanisms.

2 Particle tracking in fluorescent microscopy

2.1 Early works

An intuitive idea to solve the tracking problem is to decouple a detection stage from a linking procedure between the measured positions. When imaging cellular processes, the fluorescently labeled objects often appear as small bright spots superimposed to an uneven background. A spot is defined as a relatively small and compact object with an intensity that is both diffuse and higher than the immediate neighborhood. Detecting spots in a noisy background is a fairly standard image processing task and many efficient

methods have been designed to obtain a set of point coordinates that correspond to the objects of interest [5–8]. However in the context of living cellular systems, spot tracking is made difficult by the poor quality of images and the fact that the dynamics of spots can change over time. Moreover when the density of objects is high, the targets can aggregate temporarily which makes the appearance of spots change and their localization ambiguous.

Conventional multiple particle tracking techniques rely on a detection step of the spots followed by a nearest neighbor association (NNA) [9] or a constrained NNA [10] to build the trajectories from the set of detections. The principle is fairly intuitive: in an iterative way a trajectory is extended with the nearest detection in the next frame. For a comparative study of these techniques for tracking multiple particles in 2D images refer to [11]. These kind of methods produce satisfactory results when image sequences show a limited number of objects that are easily identifiable and move slowly. However in practice these conditions are rarely satisfied, especially when imaging fast processes in 3D. So as to keep the acquisition time as short as possible, the exposure time is generally set very short, resulting in low intensity spots and a very high acquisition noise. In this case the detection process may fail and the detection of noisy artifacts can occur due to the low signal-to-noise ratio.

In order to overcome the problem of corrupted detections, methods that do not use a frame by frame detection correspondence have been proposed to perform simultaneously detection and tracking [12]. A 2D sequence in time is considered as a 3D volume and the tracking problem is formulated as a global optimization process solved by dynamic programming. In a given track, the method looks for the best path in the spatiotemporal volume that links high intensity pixels and has minimum displacements. This method is able to extract a particle trajectory even when images are extremely noisy. However

multiple targets can be tracked only if they are sufficiently distant and can be considered as spatially separated.

A similar approach has been proposed in [13]. Here the detection of particles and the creation of trajectories are decoupled tasks but the association is modeled as a perceptual grouping problem in the spatiotemporal volume. The tracking model is defined such that each individual trajectory is a minimal path in a Riemannian metric. The proposed algorithm extracts trajectories sequentially thanks to a fast marching procedure. The method is robust to transient object disappearance and was successfully used to track blinking quantum dots on membranes in 2D images.

Both methods use a regularity constrain of the spatiotemporal volume to extract trajectories even with high levels of noise or temporary disappearance of the particles, but a number of additional assumptions are made that are not always biologically relevant. For example, a strong requirement is that particles should have a slow motion compared to the acquisition rate, so that they are nearly overlapping in two successive frames and produce smooth spatiotemporal curves in the 2D+t volume. This is not adapted to most 3D applications in which movements are fast compared to the acquisition rate, and limits the use of the methods to 2D applications. Another similar approach has been proposed in [14] where the 3D+t space is projected in a 2D space called kymograph, where trajectories are extracted as smooth curves. This method has proved efficient on data sets where movements are linear and slow.

As a general fact, one can say that methods based on the regularity of trajectories are difficult to adapt to most biological applications. They aim at extracting long and smooth trajectories while in reality trajectories are often short and very irregular due to fast changes in motion. Moreover, prior knowledge such as the level and the distribution of noise in the images, the lifetime of trajectories, statistics of appearance or disappearance

of the particles or probability of motion changes are difficult to incorporate into such models. Also, as their formalism lacks generality and flexibility, these methods are difficult to transpose out of the dedicated context they were developed for. In the sequel, we focus on the Bayesian filtering approach, which is a more general framework based on statistical modeling, and was first developed in the radar surveillance and computer vision communities.

2.2 Bayesian multiple target tracking

The Bayesian approach to tracking is inspired from the estimation theory [15] and was extensively used for years in fields such as radar surveillance [16] and computer vision. This approach consists in building the most probable trajectories given the set of detections in the entire sequence. The objective score function to maximize is defined as the likelihood of the association between tracks and detections. Corresponding probabilities are generally decomposed following the Bayes rule, which allows one to estimate the likelihood of association in an iterative way. In practice, most methods perform the computation sequentially in the order of detection occurrence, which is suitable to real time tracking applications.

In most cases trajectories can be considered independent, so the association likelihood is computed as a combination of the individual track probabilities, which are computed separately by adopting a so-called state space formalism [15, 17]. The state of a target is a vector containing its coordinates at a given time. In practice the state is unknown and we have only access to a measurement whose relationship with the state is defined in the state space by the measurement equation. The accuracy of the measurement procedure is limited by the resolution of the acquisition device and the accuracy of the localization algorithm. So in order to take into account a possible lack of precision a random term is included in the measurement equation.

The evolution equation models the dynamics of a target in the state space. The equation defines a relationship between the current and previous states. As biological targets do not show a deterministic behavior but instead exhibit a random motion such as diffusive Brownian motion, a random term, coined process noise, is introduced in the evolution equation. In order to get relevant probabilistic scores from the dynamics model, the evolution equation has to model accurately the true behavior of the target. Diffusive motion has attracted a lot of attention in the biophysical community and many subtypes have been described, each with different models of motion [18, 19]. Brownian motion, however, which is a special case of diffusive motion, is the most used in the biological tracking community. Statistical physics has shown that the position of a Brownian particle after a macroscopic time lag follows a multidimensional Gaussian distribution centered on the previous position [20], therefore the state evolution probability is written as a Gaussian function. The covariance matrix quantifies the motility of the particle: it depends on the time lag and the diffusion coefficient, which is influenced by physical data such as the shape of the target, its weight, the temperature and the viscosity of the medium.

Biological objects often move along a preferred direction when they evolve in an active flow or when they interact with the cytoskeleton, like, for instance, cargos on microtubules that move in a linear fashion with a high speed. In this case, directed or curvilinear models of motion are accurate. For most biological targets though, the motion is not known *a priori*, and, moreover, it can change during the observation time lapse. Hence it was proposed in [21] to describe the target motion as an adaptive mixture of three of the most general kinds of movement: Brownian, directed and curvilinear motions. Based on the Interacting Multiple Model (IMM) filter [22], the weights of the model mixture self adapt to the observed motion of the target. By doing so it is possible to track targets for which there is little or no knowledge about the dynamics. The

parameters of the motion models are generally fixed based on prior knowledge about the target behavior [19]. However it is also possible to define appropriate motion models that include an online estimation technique of these parameters. A good overview of such techniques is given in [23].

Thanks to the evolution and measurement equations, the probability of a given measurement knowing all past detections generated by a target can be computed. Generally, linear equations with Gaussian noises are appropriate. In this case, Kalman filtering [15] is the optimal estimation technique. Techniques such as the extended Kalman filter [16] or particle filtering [17] are used in the non linear case to approximate probability distributions.

In the field of cellular imaging, the Bayesian tracking model was first used in [24] to track particles in 2D and extended to 3D in [25]. The principle of this approach is to use an iterative decomposition of the measurements likelihood to extend a set of active tracks frame by frame. The associations are sequentially chosen following a maximum likelihood principle: the most likely association between a track and a detection is selected, then the associated measurement and track are removed from the association problem. The procedure is repeated until there are no more tracks or detections to associate. The measurements that remain unassigned are used to instantiate new tracks, while a track with no detection can be extended during some frames thanks to a prediction given by the kinetic filter. The latter possibility models the temporary disappearance of a particle which is especially important when processing noisy images or when tracking blinking objects such as quantum dots. This algorithm has been used successfully in 3D biological applications such as HIV-1 virus tracking [26] and Golgi units tracking in the *Drosophila* oocyte [27]. It is worth pointing out that 3D imaging facilitates the tracking as it reduces the occlusion problems that occur in 2D images.

Particle filtering has been proposed in [28, 29] as an alternative resolution scheme for the Bayesian tracking problem in 2D images and used in [30] to track HIV-1 particles. Particle filtering is a sampling technique used to model the uncertainty of the association between tracks and targets. The algorithm maintains several hypotheses of association called particles in order to implicitly delay the association decision to a latter time. The selection of association hypotheses is achieved by a probabilistic resampling of the particles at each frame. An up-to-date review of particle filtering techniques can be found in [31]. There are however a number of critical issues like the selection of the optimal number of particles, the sample impoverishment or the drift of particles that can make the technique difficult to apply. A number of recent improvements proposed by the computer vision community [32–34] or specific to the biological field, like the introduction of multiple models of motion [35] have made the method more suitable for biological tracking applications.

The Bayesian framework is still very attractive for particle tracking in biology, as exemplified by recent works [36, 37]. In [36] Bayesian filtering was combined with a post processing technique to link incomplete tracks and obtain very long 2D trajectories of mRNA molecules in cells. In [37] the Bayesian tracking approach was used to study the *in vivo* colocalization of Oskar mRNA and trans-acting proteins.

While they have allowed significant improvements in the modeling of multiple particle tracking systems, the described Bayesian approaches do however suffer from a number of limitations in biological imaging. In particular, as they fully depend on the kinetic models of targets, which in many real situations are not discriminant enough or lack robustness, the association performances are often sub-optimal. For instance, in the frequent case of a high density of similar and closely spaced targets, subjected to Brownian diffusion, the association scores based on kinetic parameters are often not able

to provide the true associations. Indeed, because of the randomness of the motion process, many hypotheses in the association problem have similar kinetic scores and it often happens that the true association hypothesis is not the one with the best kinetic score. In such cases, decisions based only on the kinetic information therefore lead to false associations that have a dramatic impact on the downstream track analysis.

One proposed solution to this problem has been to use the intensity of the detected objects in the Kalman filtering technique [35, 36, 38]. The state space representation allows the extension of the measurement and state vector with a number of non kinetic features. The main difficulty of this kind of approach is to fuse this additional feature information with the kinetic information in a proper way. A probabilistic model of temporal evolution and a measurement model for non kinetic features are indeed required, and it is a major issue to design a balanced score function of the kinetic and the non kinetic information. Some of our recent work [39, 40] proposes a solution to these issues by integrating image and kinetic information in a statistical framework.

2.3 Joint image and kinetic based tracking

Tracking numerous and closely spaced targets is a very challenging task because of the limitations of the kinetic information and the interactions between targets. In order to solve this issue we have presented in [39] a unified Bayesian framework that fully exploits all the information available about the scene. We revisited the original definition of the scene likelihood, in order to define an enriched likelihood of association that combines in one function the target motion and the image formation process description, hereafter called appearance information.

The kinetic information and appearance information are split following the Bayes rule. On one hand the kinetic part is computed thanks to a Kalman filtering-derived technique

and using multiple models of motion. On the other hand the appearance likelihood relies on the description of a statistical model of image generation, which depends on the acquisition device. In practice this process is known accurately since the optical study of microscopy devices is a very active research field and accurate models exist [41, 42]. We describe an image at a given time as the sum of the intensities originating from the targets, of a locally constant background and of a random acquisition noise. The noise is a random value that follows a probabilistic distribution that is generally well characterized, so its distribution will be used to give an accurate score to associations. The difference between the observed image and the profiles and the background, which we call the residual, should follow the same distribution as the noise if the association is correct. Hence we use the statistics of the residual to compute the likelihood of an association given the image.

The appearance model is also used to split closely spaced targets that appear fused due to the resolution limit. The procedure is naturally integrated in the tracking procedure without any *ad hoc* decision step. Super resolution techniques [6, 43] also exploit the appearance of objects to address this issue, although the approach introduced in [39] performs significantly better, especially when the level of noise is high. This higher sensibility and robustness of the method comes from the exploitation of all the information available. Data such as dynamics of targets, statistics of targets appearance and disappearance, are used to decide if spots have to be separated. This method combined with the IMM filter was used to track in 3D hundreds of small fluorescent beads diffusing in water [40]. Figure 1 depicts the trajectories extracted from this very dense environment where beads have a fast and directed motion due to a global flow. It also shows that the separation of aggregated particles is efficient.

3 Motion analysis and applications

3.1 Trajectories analysis

The final stage consists in the analysis of the trajectories resulting from cell or particle tracking. The exact type of analysis depends intrinsically on the biological question, but the study of the diffusion is most of the time a relevant issue. The Mean Square Displacement (MSD) [18, 19] is a useful measure to characterize the diffusion of individual particles. By definition, the MSD is a function of the time lag during which displacements are measured and averaged. The shape of the MSD-time curve for a given trajectory is indicative of the mode of motion of the corresponding particle. We summarize the behavior of the MSD curve for different types of motion in Figure 2. In the case of normal diffusion by pure thermally driven Brownian motion, the MSD will increase linearly as a function of time. The diffusion constant determines the slope of the MSD line. In the case of flow or active transport the MSD increases more rapidly: quadratically with the time lag instead of linearly. The case of anomalous subdiffusion, characterized by a lagging MSD-time curve compared to normal diffusion, occurs if the motion is obstructed by obstacles. Confined motion, caused by corrals, tethering or other restrictions, manifests itself by a converging curve, where the limiting MSD value is proportional to the size of the region accessible for diffusion. Once the type of motion is identified, some subsequent parameters can be computed by regression. For instance the Brownian motion is fully characterized by its diffusion coefficient, and a directed motion is described by its direction and the velocity along it.

3.2 3D tracking of viruses

In [26], 3D tracking MSD analyses were performed in order to study virus-host cell interactions during infection of human cells by HIV-1 virus. The integrase of the virus was labeled with the bis-arsenical fluorescein derivative FlAsH which allowed imaging

both intracytoplasmic and intranuclear HIV-1 complexes. Tracking experiments of virus complexes resulted in the construction of 923 trajectories through time in 3D. The MSD analysis was used to determine the types of movement of HIV-1 complexes in the cytoplasm, during the association with the nuclear membranes and in the nucleus. For cytoplasmic HIV-1 complexes at early time points after infection, the shape of the MSD curve was a polynomial of order 2 which indicates a directed motion. Two types of such directed motions were distinguished based on their characteristics and velocities. First some fast and curvilinear motions (82 +/- 7.4 *nm*/s) exhibited a saltatory property: both anterograde and retrograde movements were observed but with a global directionality towards the nucleus. This feature is compatible with microtubule-associated movement. The second class of motion was observed in the perinuclear area. It was still a directed motion but with lower speed (18+/- 1.3 *nm*/s) on short distances, which is typical of actin-based transport. The implication of microtubules and actin in the motion of virus complexes was then confirmed by other standard biological techniques. For instance colocalization studies showed that HIV-1 complexes overlapped with both microtubules and actin filaments. Then pharmacological experiments were done: the dynein-based transport was uncoupled with a specific drug which resulted in the inhibition of entrance of the labeled virus into the nuclear compartment, while the inhibition of the actin-mediated transport resulted on a drastic diminution of cytoplasmic transport of viruses. The dynamic study of association of complexes with nuclear membranes revealed significantly different characteristics compared to cytoplasmic transport. For instance, once the virus complexes were associated with the membrane their MSD trajectory curve was indicative of a restricted motion which implied confinement to a small volume or tethering.

3.3 Prion tracking inside tunnelling nanotubes (TNTs)

A recent study [4] used latest advances in particle tracking methods [44] to characterize the intercellular spreading of prions. Imaging techniques were used to show that TNTs allow transfer of exogenous and endogenous prions between infected and naive neuronal CAD cells. The CAD cells were labeled with LysoTracker red and imaged by spinning-disk confocal microscopy in 3D. Lyso-Tracker-positive vesicles were visualized moving towards naive cells inside nanotubes. The infectious vesicles were automatically detected, and their trajectories were reconstructed thanks to a Bayesian tracking method [44], while moving inside TNTs and entering into the cells. A typical 3D prion vesicle trajectory is shown in Figure 3. The MSD analysis of the track revealed that the vesicle has a directed motion towards the recipient cell. The estimated velocity of the tracked vesicle (41.*5 nm/s* on average) is very similar to that calculated for a complex myosin VI and the GLUT1 transporter binding protein, indicating the possible involvement of actin mediated motors

4 Conclusion

Recent advances in optical systems and imaging protocols have allowed observing pathogens over time at the cellular and sub-cellular scale in multiple colors and dimensions in a fast and automated way. Systematic quantification of the recorded data sets requires fully automated image processing tools and algorithms specifically adapted to the problem at hand. Throughout this review we have browsed a number of methods developed to track sub-cellular objects in image sequences. The particle tracking problem includes many issues such as particles localization, motion estimation and measurements linking. The definition of the Bayesian framework has been an important achievement in the modeling of such a complex problem since it allows one to include in a uniform way prior knowledge about the scene and to define general objective functions. Enriching this framework with even more models dedicated to bioimaging and

developing algorithms able to solve problems in this formalism should attract much interest in the future.

5 References

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Figure legends

Figure 1

Fluorescent beads (200 nm diameter) in water imaged with a Disk Scanning confocal microscope [50]. Left: Trajectories extracted by joint image and kinetic tracking. Right: Separation of targets by the tracking algorithm while spots appear fused at frames $t13$ and $t14$.

Figure 2

Mean Square displacement curve for three different types of motions: pure diffusive (green), super diffusive (red) and sub diffusive (blue). The type of motion is related on the curvature of the curve.

Figure 3

Prion tracking on tunnelling nanotubes. Left: 3D view of a prion vesicle moving on a TNT towards a naive CAD cell. Right: Mean Square Displacement analysis of the vesicle trajectory inside the TNT (blue), and entering into the cell (green). The curvature of each curve indicates a directed motion with different velocities: the infectious vesicle exhibits a fast motion inside the TNT (41.5nm/s on average), and a slow motion (around 10nm/s) in the cell. From [10].

Jean-Christophe Olivo-Marin is the head of the Quantitative Image Analysis Unit at the Institut Pasteur, Paris, France. His has a long experience of multi-disciplinary approaches in biological imaging, and his research interests are in image analysis of multidimensional microscopy images, pattern recognition and motion analysis for cellular dynamics studies.

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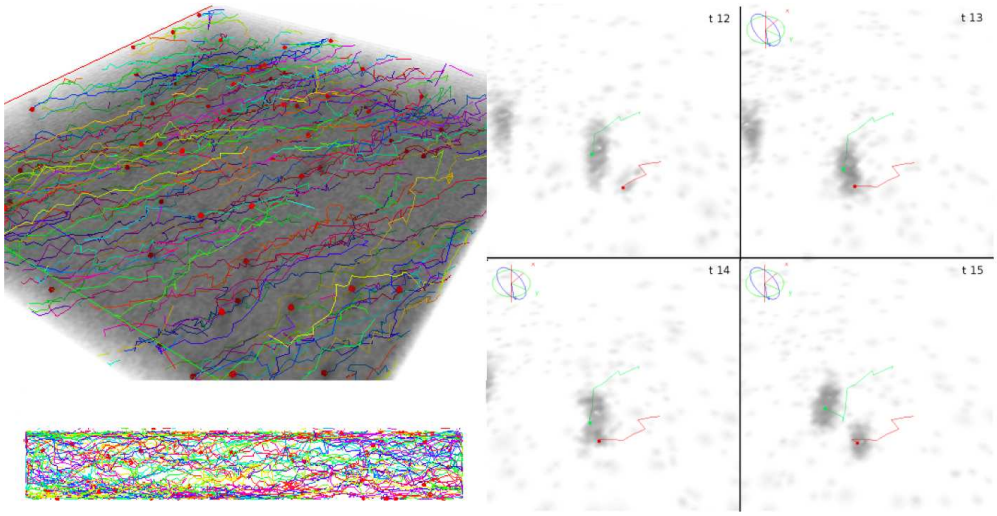
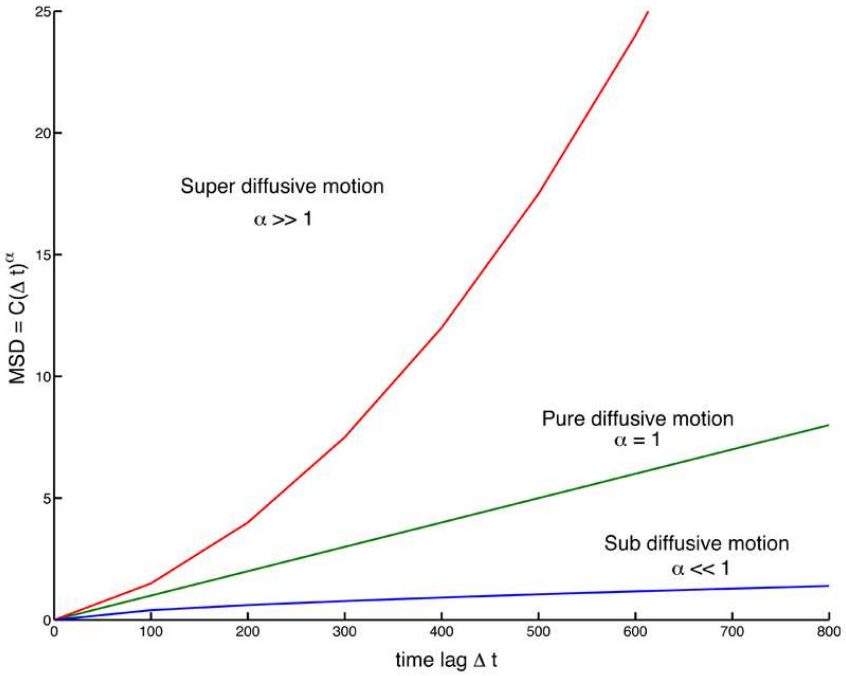
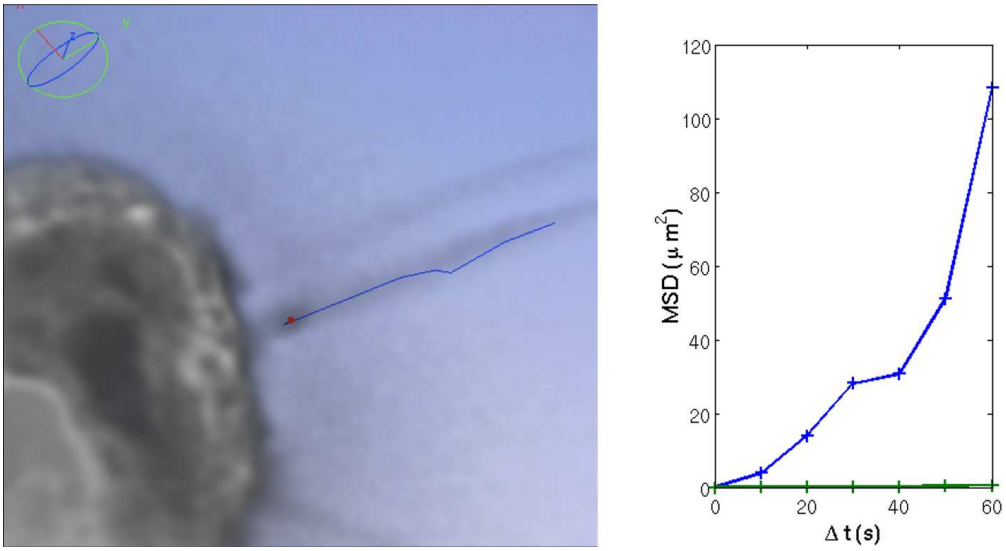


Fluorescent beads (200 nm diameter) in water imaged with a Disk Scanning confocal microscope [50]. Left: Trajectories extracted by joint image and kinetic tracking. Right: Separation of targets by the tracking algorithm while spots appear fused at frames t13 and t14
120x60mm (300 x 300 DPI)



Mean Square displacement curve for three different types of motions: pure diffusive (green), super diffusive (red) and sub diffusive (blue). The type of motion is related on the curvature of the curve
80x59mm (300 x 300 DPI)



Prion tracking on tunnelling nanotubes. Left: 3D view of a prion vesicle moving on a TNT towards a naive CAD cell. Right: Mean Square Displacement analysis of the vesicle trajectory inside the TNT (blue), and entering into the cell (green). The curvature of each curve indicates a directed motion with different velocities: the infectious vesicle exhibits a fast motion inside the TNT (41.5nm/s on average), and a slow motion (around 10nm/s) in the cell. From [10]
119x65mm (300 x 300 DPI)