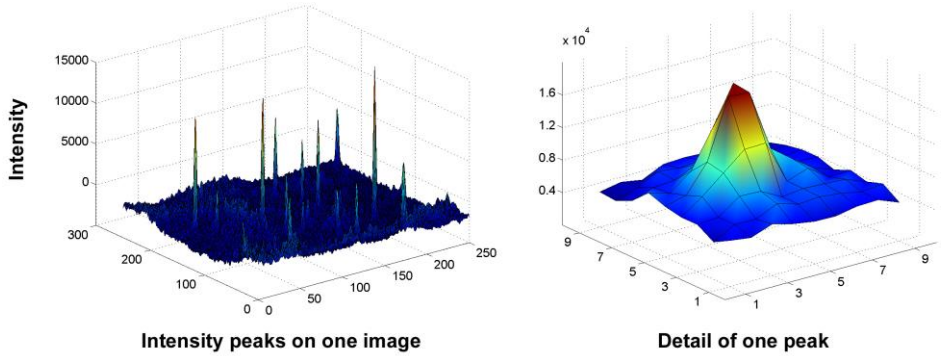
Insights into SPT procedures

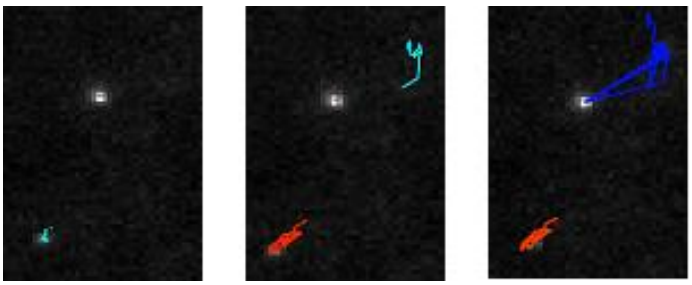
1) Detection of particles and initial tracking

Once detected by cross-correlation, the fluorescent signals (peaks) are fitted to a two-dimensional Gaussian surface. This allows their sub-wavelength positioning, typically below 50 nm for single dyes and around 10 nm for quantum dots. The shape (width, intensity) of the peak is compared to cut-off parameters; the peak is discarded if it does not show the characteristic shape corresponding to one single particle (intensity and width comparable to that of the expected PSF).



Intensity peaks on one image **Detail of one peak**

The two-dimensional trajectories of single particles are constructed by correlation analysis between consecutive images. This method connects pairs of intensity peaks (spots) detected in two successive images by minimizing the deviation of the mean diffusion constant of the connected spots with respect to a given initial diffusion constant (**initial D**). In other words, the trajectory of one molecule will add one point if there is a fluorescent spot within a given distance from the position of the previous spot. The distance is calculated in base of an initial diffusion coefficient. These parameters must be correctly chosen: a value too big of initial D can lead to wrong connections between different objects and conversely, a initial D value too small will cause loosing of the molecules that move fast.



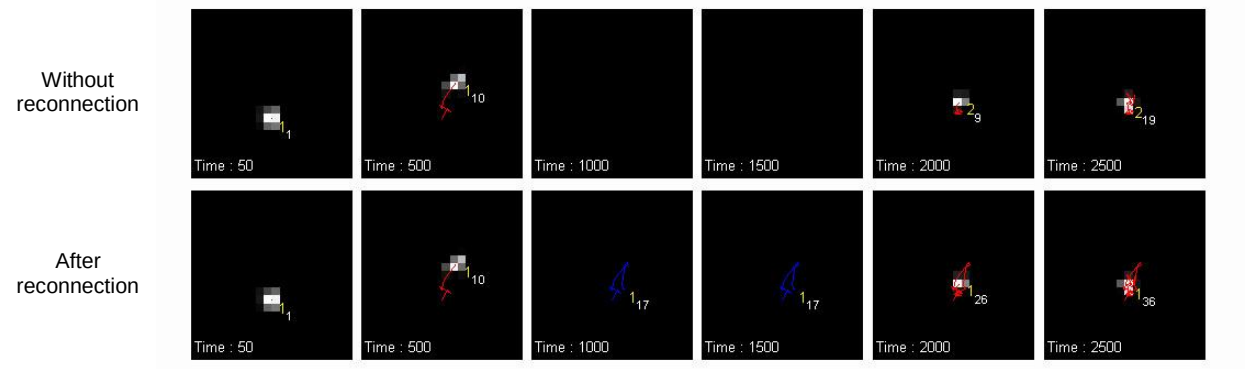
The tracking results of the same quantum dots using different values of initial D. With D=0.001 (left), the trajectories are short, and the faster particles are lost. D=0.04 (center) shows a correct tracking. D=1 (right) caused wrong connections between different particles.

2) Connecting the initial trajectories

The initial trajectories are left short on purpose to increase computing efficiency. In addition to this, there may be several trajectories constructed for a single fluorescent particle because it went out of focus or it displays anomalous fluorescence properties (for example, reversible photobleaching or "blinking"). Therefore, it is necessary to re-connect trajectories created by the initial tracking but that were tracked separately.

To allow the connection, the algorithm uses criteria in time (number of images without signal) and space (the distance roamed during blinking). More precisely, in each iteration, the last position of the molecule of the first trajectory is connected to the first position of the closest following trajectory, only if it is within a time and a spatial limit. The same procedure is used for all trajectories and repeated iteratively until no connexions are allowed.

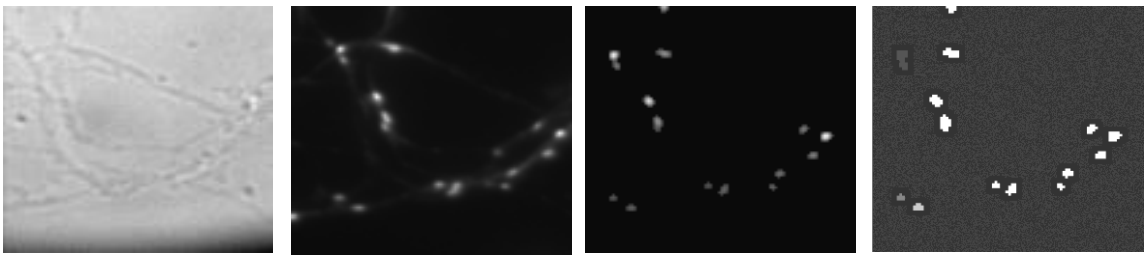
The parameters of reconnection (maximum distance for the following position and maximum 'off' time) must be adjusted empirically.



Example of reconnection. The blue trajectory indicates the blinking period. The number in yellow is the number of the trajectory and the number in white, the amount of points of the trajectory at this image.

3) Localization

Localization images are masks in which all the pixels are black apart from those indicating the presence of "domain(s)". Mask can be binary images or not. The stained zones (domains) are recognized as the clear objects remaining. They have to be prepared before (using ImageJ for example). Each zone is numbered for trajectory analysis. All the other pixels have 0 value.



DIC **Synaptic staining** **After cleaning** **Automated detection**

Next step is to assign the localization of each trajectory depending on its localization inside domains ("in") or outside ("out") domains. If the trajectory goes in/out a given area, it will be chopped to separate segments "in" and "out" the domain.