Practical diffusion analysis

MSD, diffusion coefficient, dwell time, confinement… The results of SPT are not always easy to digest. Let see some hints to survive.

☺ *Marks practical suggestions…*

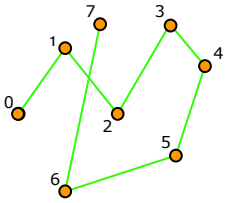
Note: These are important remarks.

1) The basics: Mean Square Displacement (MSD) and diffusion coefficient (D)

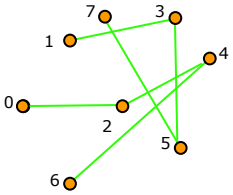
The analysis of the movement on a surface is done constructing the MSD plot vs the intervals of time. Note that increasing the interval of time means to decrease the number of displacements (steps) used to calculate the MSD, which will consequently increase the error of the calculation:

$$MSD(n\,\tau) = (N-n)^{-1} \sum_{i=1}^{N-n} \left((x_{i+n} + x_i)^2 + (y_{i+n} + y_i)^2 \right)$$

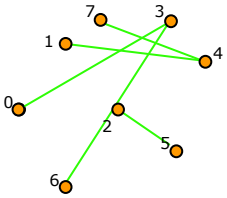
τ : time between images
N: number of trajectory points



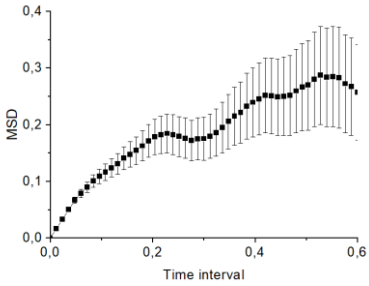
Time lag = 1
(7 steps)



Time lag = 2
(6 steps)

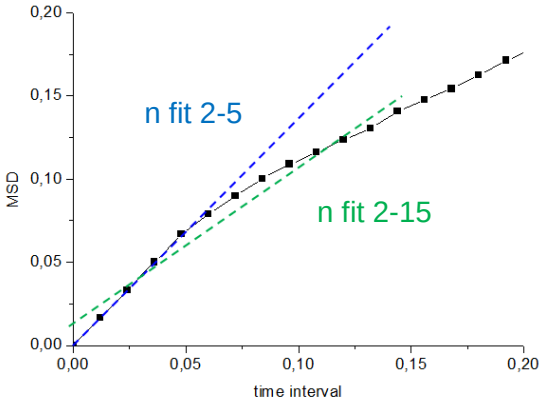


Time lag = 3
(5 steps)



The diffusion coefficient D is defined as the initial slope of the MSD plot (assuming that the beginning of the plot is still in a linear regime of Brownian diffusion): *MSD= 4 D t*

Note: The number of MSD points (nb_fits) considered to calculate D are set in the window for diffusion analysis (default: 5, and the fit is done between points 2 to 5). If you increase this number, you may obtain different values of D if the MSD is not linear, as shown in the figure:



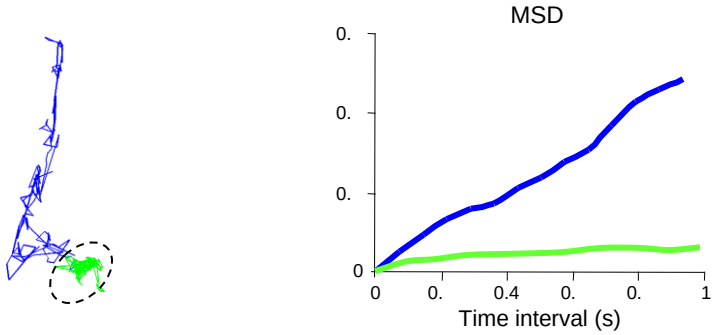
The longer the interval of time, the smaller number of steps can be used to calculate the MSD and MSD plot becomes very noisy at long time intervals. As a rule of thumb, we can trust the calculation up to ~30% of the total length of the trajectory. Therefore, to calculate D with a minimum of precision, the trajectory must have at least (nb_fits * 3) points.

Note: The minimum number of points is set on the main window, '**Min length**' (default: 15)

Note: There is a finite pointing accuracy given by the signal-to-noise ratio. Therefore, it is impossible to get D=0 for an immobile particle. The pointing accuracy has to be measured (tracking immobile particles) to set an immobility threshold.

If the localization over domains was done, trajectories are sorted into those happing outside domains (*out*) and those colocalizing with domains (*in*):

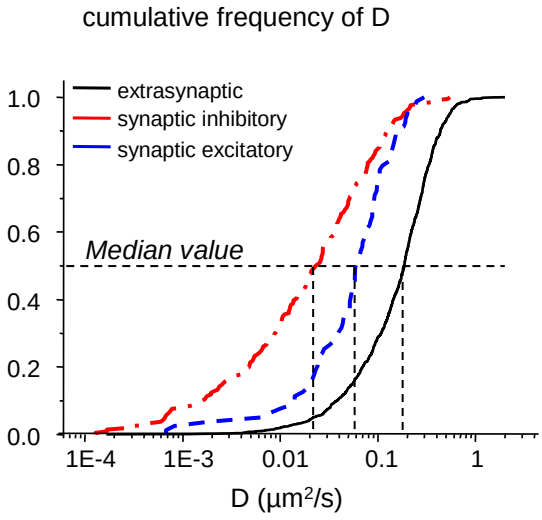
Example of trajectory that switches between "in" (green) and "out" (blue) localizations:



In this example, the mobility "in" the domain is lower that "out".

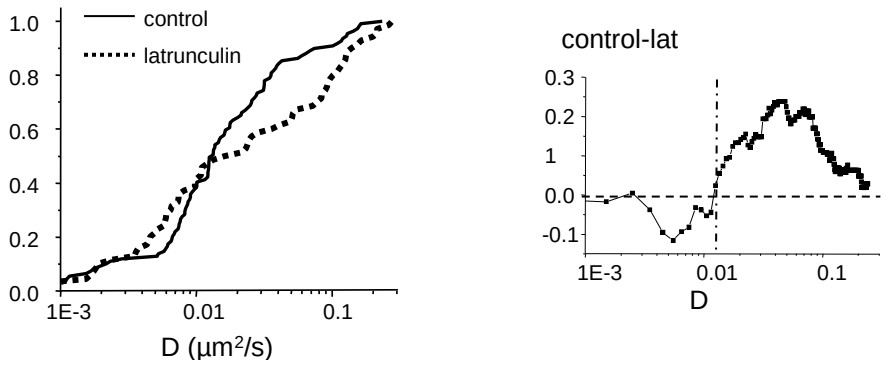
The distributions of values of D usually span several orders of magnitude and do not follow a normal distribution. Thus, it is preferable to use median values instead of the mean to compare D between different experiments.

A convenient way to display the distribution of D is to use cumulative frequencies:



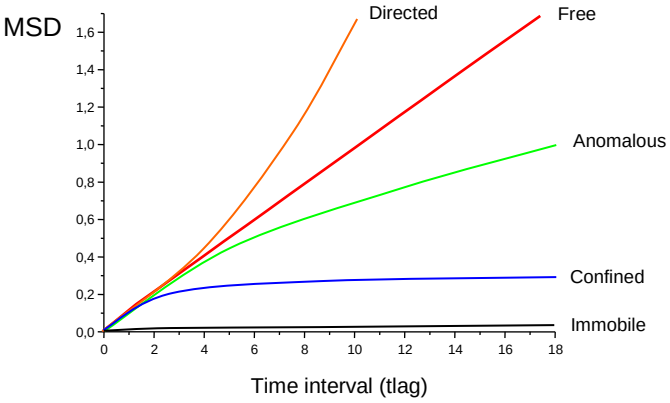
A shift of the distribution to the left means less diffusivity. The statistical analyses that can be done are the analysis of the medians (Mann-Whitney test, MW) or the comparison of the distributions (Kolmogorov-Smirnov test, KS).

☺ Inside the population of particles, there can be subgroups with different mobility that may be affected differently. In the following example, the application of latrunculin only affected the faster molecules. Therefore, the median values are similar although there is an effect of the drug. This can be better shown using a subtractive histogram:



2) Diffusion behaviour: when the MSD forgets Mr. Brown

In the case of pure Brownian movement, the dependence of the MSD vs the interval of time is linear. We can expect that at the nanoscopic level, all the molecules display Brownian motion. However at the microscopic level, as they experiment other forces, the presence of obstacles, etc, their movements are not longer random. As a consequence, the MSD plot deviates from linearity. Therefore, the diffusion behaviour of the particle can be extracted from the shape of its MSD:



Free:
MSD = 4 D t

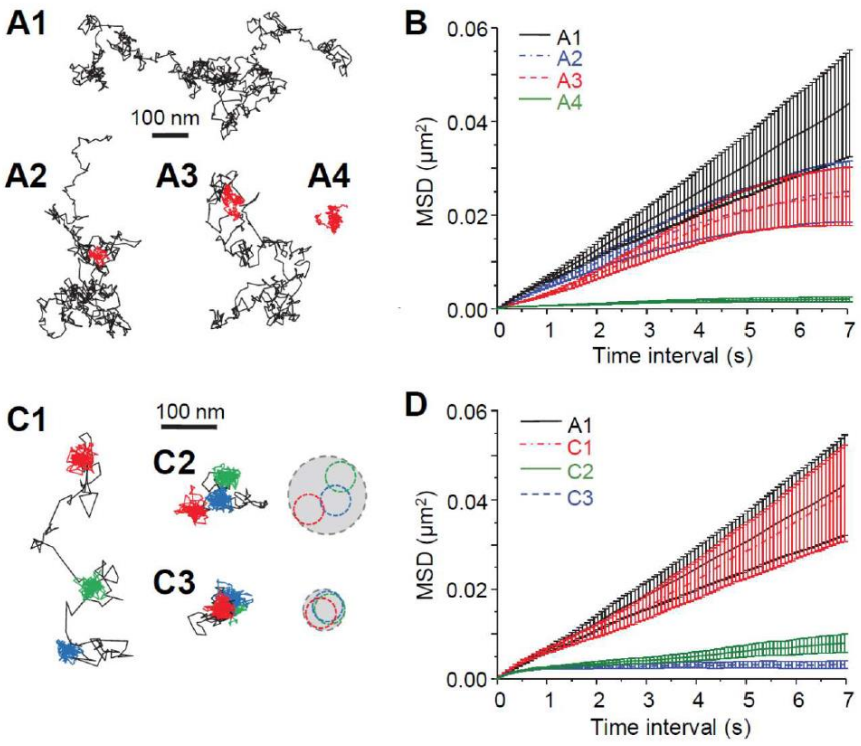
Anomalous:
MSD = 4 D t^{α} ($\alpha<1$)

Confined:
MSD = $L^2/3$ (1-exp(-12Dt/L^2))
L: size of the confinement area

Directed:
MSD = 4 D t + $(V t)^2$

ATTENTION! : The averaging effect of MSD calculation overlooks transient confinement periods

The MSD provides the simplest type of classification of the diffusion behaviour. Unfortunately, the information obtained from MSD analysis is limited and, in practice, different situations of non-Brownian diffusion can produce the same MSD. This is due to the fact that the MSD is calculated by averaging all the displacements within the trajectory that correspond to a given time interval. Thus, if the molecule switches between periods with different diffusive behaviours, the final MSD depends not only from the difference in diffusivity but also from the duration of each period:

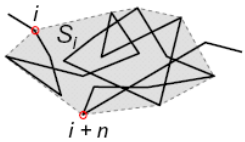


(A) Brownian simulated trajectories without (A1), with one period of confinement (in red) of 3 s in a confinement area of 50 nm in diameter (A2), with one period of confinement of 5 s in a confinement area of 100 nm in diameter (A3) or always confined in an area of 100 nm in diameter (A4). (B) *MSD* values of the simulated trajectories in (A) (MSD ± s.e.m.). (C) Brownian simulated trajectories with three periods of confinement (in color) in 30 nm diameter areas that were distanced (C1), apposed (C2) or co-localized (C3). On the right: minimum circles containing each confinement period (in colors) or the whole trajectory (grey). (D) *MSD* values of the simulated trajectories in (C) and of trajectory A1 for comparison (MSD ± s.e.m.).

3) Packing coefficient

Reference:
Renner M, Wang L, Levi S, Hennekinne L, Triller A. (2017) A Simple and Powerful Analysis of Lateral Subdiffusion Using Single Particle Tracking. Biophys J. 113:2452-2463.

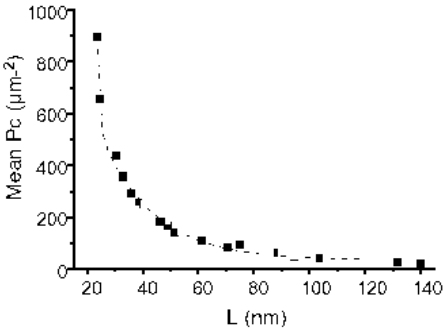
In order to overcome MSD's drawbacks, we proposed a new analysis parameter: the packing coefficient *Pc*. This parameter was defined to analyse transient changes in diffusion behaviour, and it is calculated at each time point *i* as



$$Pc_i = \sum_i^{i+n-1} \frac{(x_{i+1}-x_i)^2+(y_{i+1}-y_i)^2}{S_i^2}$$

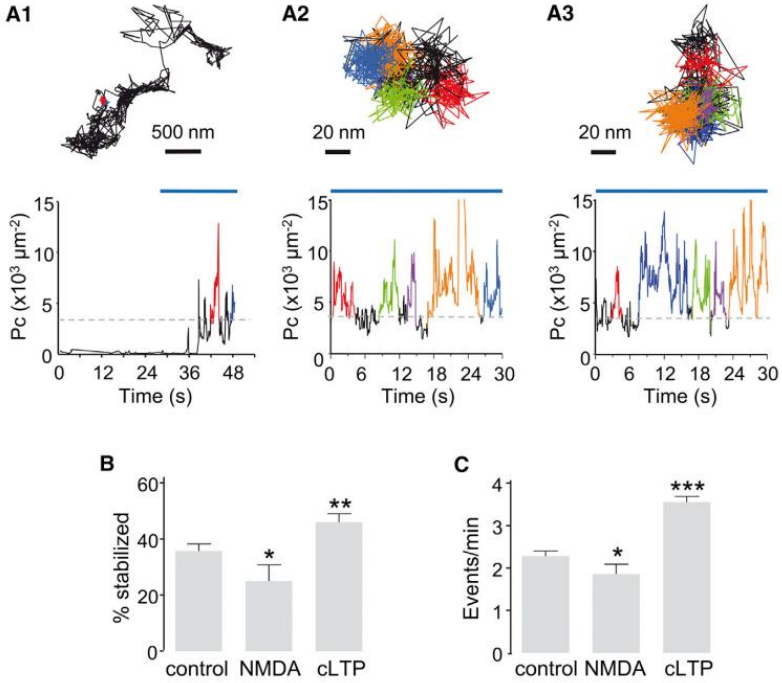
where x_i, y_i are the coordinates at time *i*; x_{i+1}, y_{i+1} are the coordinates at time *i+1, n* is the length of the time window and S_i is the surface area of the convex hull of the trajectory segment between time points *i* and *i+n:*

Pc scales with the size of the confinement area (L); thus it is possible to identify periods of confinement by setting a threshold corresponding to a given confinement area size.



Transitions between diffusive states

If a threshold can be determined to detect stabilization periods with high confinement (i.e. immobilizations or “stabilizations”), it may be interesting to evaluate the amount of these periods and their duration:



(A) Examples of Pc values upon time (Renner et al 2017). The detected periods of stabilization are shown in color. The horizontal blue line shows the localization in a domain (synapses) in time. The horizontal discontinuous line shows the threshold of Pc. Example of results for the percentage of stabilized trajectories (B) and for the frequency of stabilization events (number of events per min, C)

This analysis is particularly useful when the transitions imply a quasi-immobilization by interactions with immobile molecules. Due to the limited localization accuracy in SPT, immobilisation is translated into confinement in an area whose size is the localization accuracy. In this way, the Pc analysis is able to identify periods of transient immobilization. Assuming that these periods arise from a scaffolding interaction, the effective k_{on} and k_{off} of this interaction can be extracted from the frequency and the duration of immobilizations, respectively (Renner et al 2017). The analysis of transitions, using Pc, provides these values after sorting trajectories by transitions, as well as other parameters to characterize the level of heterogeneity in diffusivity of the population of molecules.

3) Dwell time

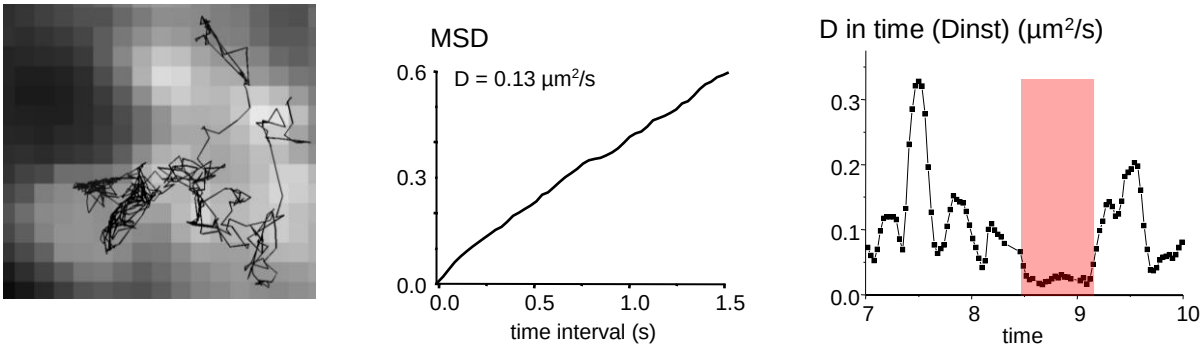
We call *dwell time* or *residency time* the time that a particle spends in a given localization. Typically, we measure the dwell time at membrane domains such as synapses. It can be given in time units (s) or as the percentage over the total time of the acquisition, divided by the times the particle exits the domain.

This apparently easy calculation is in fact rather complicate due to technical reasons. In most cases, the acquisitions are too short to have a significant number of particles that enter and exit a given region. Therefore, the dwell time significantly depends a lot on the total length of the acquisition.

4) Diffusion 'point to point': D vs time

As mentioned above, the diffusion coefficient is defined as the initial slope of the MSD vs time interval plot. There is only one value for the whole trajectory, independently if the particle stopped for a while and then started moving again. In fact, D is a representative value of the diffusivity of a particle if it does not change significantly its diffusion behaviour… A way to analyze the diffusion point to point and reveal these changes in diffusion is not calculate D over a portion of the trajectory (sliding window) to have an instantaneous D (*Dinst*) value for each point of the trajectory.

Note: the fact that D is the initial slope of the MSD vs tlag plot does not mean it corresponds to the beginning of the trajectory!



Example of a trajectory that displays changes in mobility. The MSD is linear and D is 0.13 µm²/s. However, the calculation of Dinst shows that the particle 'stopped' for a while (pink region).

To proceed with this analysis, you have to specify the size of the trajectory portion (**Size sliding window for MSD calculation)**.

Note: Dinst is calculated over a portion of trajectory that has to be long enough to provide a good MSD! Set a sliding window small enough to have 'local' measurements of D but long enough to have a good calculation of D… Be aware that you will not be able to detect short lasting changes.

As a result, there will be '-dinst.txt' files saved in \Dinst folder:

gav02sdinst

	Trajectory[X]	Frame[Y]	x[Y]	y[Y]	loc[Y]	loc2[Y]	Dinst[Y]	angle[Y]
1	1	1	127,45871	91,64334	0	0	0,00318	-1,79139
2	1	2	127,41445	91,63341	0	0	0,00208	-2,51609
3	1	3	127,30888	91,48724	0	0	4,83111E-4	-2,86541
4	1	4	127,30161	91,46159	0	0	6,74213E-4	2,70295
5	1	5	127,36097	91,33506	0	0	0,001	-0,75032
6	1	7	127,29957	91,40093	0	0	0,00121	2,38631
7	1	8	127,3243	91,37466	0	0	0,00125	-0,05309
8	1	9	127,31722	91,50789	0	0	4,34827E-4	0,05162
9	1	10	127,31814	91,5257	0	0	6,30133E-4	0,2499
10	1	11	127,34049	91,61326	0	0	0,00121	3,02985
11	1	12	127,35322	91,49981	0	0	0,00151	-1,29082
12	1	13	127,33061	91,50632	0	0	8,88579E-4	0,30334
13	1	14	127,385	91,68008	0	0	8,88622E-4	2,04864
14	1	15	127,48709	91,62722	0	0	0,00132	-1,25999
15	1	16	127,35435	91,66985	0	0	0,00133	1,45022

X and Y are the positions of the trajectory, loc and loc2 have the information about localization (optional), Dinst is the D in time and the angle is the direction of the next displacement.

Other useful reading

Bannai H, Levi S, Schweizer C, Dahan M, Triller A (2006) Imaging the lateral diffusion of membrane molecules with quantum dots. Nat Protoc 1:2628-2634.

Ehrensperger MV, Hanus C, Vannier C, Triller A, Dahan M (2007) Multiple association states between glycine receptors and gephyrin identified by SPT analysis. Biophys J. 92:3706–3718.

Kusumi A, Sako Y, Yamamoto M (1993) Confined lateral diffusion of membrane receptors as studied by single particle tracking (nanovid microscopy). Effects of calcium-induced differentiation in cultured epithelial cells. Biophys J 65:2021-2040.

Triller A, Choquet D (2008) New concepts in synaptic biology derived from single-molecule imaging. Neuron 59:359-374.