ONI

TECHNICAL GUIDE

Getting started with Single Particle Tracking (SPT)

Single-particle tracking (SPT) is a powerful technique that follows the mobility of individual particles in living cells or within a medium. Single particles can include small molecules, proteins, virions, or vesicles, and can provide critical insight into the dynamic behavior of individual molecules or large macromolecular complexes.

1. When to choose SPT

Benefits
SPT allows researchers to determine the trajectories, mobility, behaviors and interactions between molecules over time. SPT is usually measured by labeling the particle of interest with a fluorescent dye and imaging that particle over time while recording its motion continuously to create a trajectory, which can be quantitatively analyzed for its velocity, diffusion coefficient, displacement, distance traveled and mean speed.

SPT vs FRAP
Tracking the movement of endogenous proteins can uncover the heterogeneity in behavior of proteins including how many are immobile vs diffusing and how long they are immobile for. This is often a complementary technique to fluorescence recovery after photobleaching (FRAP) which is the classic method for measuring dynamics. In FRAP, you photobleach an area of the cell with your strong laser power and see how fast it recovers, but as this is a bulk technique it is difficult to assess the different populations of molecules and this is why single particle tracking has become a popular technique.

2. Choosing the best tag or fluorophore

Particles to be tracked can be labeled with different fluorescent probes including organic dyes, fluorescent proteins (FPs), dye-labeled antibodies, and nanocrystals such as quantum dots. In SPT, it is the fluorophore attached to the particle that is followed over time. When choosing the fluorophore, consider its size, the location of the target to be labeled (intracellular/extracellular or non-cellular), the image acquisition settings and the labeling method.

Once you have chosen the protein to track, you need to select your tag. A tag is needed because it is very difficult to label proteins with antibodies without affecting their function and if your protein is inside the cell it will be more difficult to label.

Self-labeling tags
HaloTag®: The gold standard tag for SPT is the HaloTag® (Promega). This tag rapidly covalently binds to its ligand, which can be attached to any synthetic fluorophores. These dyes are brighter and more stable than photoactivatable fluorescent proteins, which have been traditionally used in the past for SPT. They also offer increased versatility and photostability.

Commercially-available kits can be used to label proteins on the extracellular face of cells, purified proteins, antibodies and oligonucleotides using modified dyes.

Tagging proteins at their termini can severely impair their function. The HaloTag and FPs are roughly 30 kDa, and can have a considerable steric effect if they are located near to an important domain.

Tip! It is important to check in the literature if previous work has verified where to tag your protein of interest. Otherwise find out how evolutionarily conserved the protein's termini are by performing a multiple sequence alignment, using Uniprot to download protein sequences and Jalview to run and visualize the alignment.

SNAP®-Tag: We recommend using the HaloTag for single-color imaging and a combination of the HaloTag and SNAP-Tag for two-color imaging. SNAP-Tag ligands are a bit less cell-permeable than the Halo ligand.

Other similar genetically encoded tags have been developed to permeate live cells include FLAG™- and CLIP®-tags, which are also gaining interest and becoming widely available.

Cell-permeable SPT fluorophores (for intracellular labeling)
We are highlighting dyes developed in Luke Lavis' lab and distributed by Promega, Tocris and Janelia. There are two types of dye that can be used for SPT with different mechanisms to achieve sparsity.

1. Dyes that are commonly used, typically start off fluorescent and then blink under high laser power powers. Janelia Fluor® (JF) 549 (JF549) would be one of the best and a good one to start out with for initial experiments. JF646 is another good one to combine it with for two-color imaging.

2. Photoactivatable (PA) dyes, PA-Janelia Fluor® 549, PA-Janelia Flour® 646, which start off in a dark state and can be turned on using the UV laser. This gives you more control over the density of the spots.

3. Others to use: TMR conjugates (TMR-star). Here is a full list of available fluorescent dyes for HaloTag®

Cell-Impermeant Ligands (for cell-surface labeling)
Two examples of cell-impermeant dyes are Alexa Fluor® 488, which has a similar spectra to Oregon Green®; and Alexa Fluor® 660, which is a suitable far-red dye that is water soluble and pH-insensitive between pH 4 - pH 10.

Photoactivatable fluorescent proteins
Whilst fluorescent proteins are relatively small and label with high specificity, they can interfere in protein folding and substrate binding dynamics, emit far fewer photons and are less stable than organic dyes[1]. Two commonly used examples are PA-GFP or PA-mCherry.

FPs fused to proteins of interest can be introduced into cells on a plasmid vector via transfection for transient expression or packaged into viruses for stable expression via transduction.

How to tag your protein of interest
Protein overexpression can detrimentally impact cellular functions, such as overloading biological pathways, aggregating proteins, and disrupting the regulatory functions of the target protein.

The best method for performing SPT of cellular proteins is to use CRISPR-Cas9 technology to knock in the tag adjacent to the gene encoding the endogenous protein to avoid any biological effects of recombinant protein overexpression.

3. Labeling considerations

Fluorophore size

- Steric hindrance from an attached fluorophore may interfere with the mobility and/or biological functions of some particles. This inhibition from steric hindrance can sometimes be corrected by incorporating a long and flexible linker between the tag and the protein of interest.

- An option for labeling cell surface markers such as transmembrane proteins in the plasma membrane is to use antibodies, antibody fragments or camelid nanobodies of various sizes to label the extracellular domain of these proteins to track their lateral mobility or internalization and trafficking inside the cell.

- All tagged particles should be verified for proper function and molecular interactions when interpreting imaging results and describing biological behaviors.

Labeling density
For SPT it is important for the fluorescent molecules to be sparse. The density of the molecules should be low enough that individual fluorophores can be resolved from each other, and that by eye it is clear where tracks end and start. If the tracks are regularly crossing over each other, the software will start linking multiple molecules into one track, giving unreliable results.

Frequently, if you image your protein of interest with standard conditions, the fluorophores are so close to each other that they cannot be distinguished nor tracked. However, the sample sample, with the same dye but imaged with higher laser power, can make a difference. This dye will "blink" when exposed to a high imaging laser. While some of the molecules are bleached permanently, a large fraction of them go into a temporary dark state. They then turn back on at a rate that allows us to distinguish the individual molecules and track the individual spots.

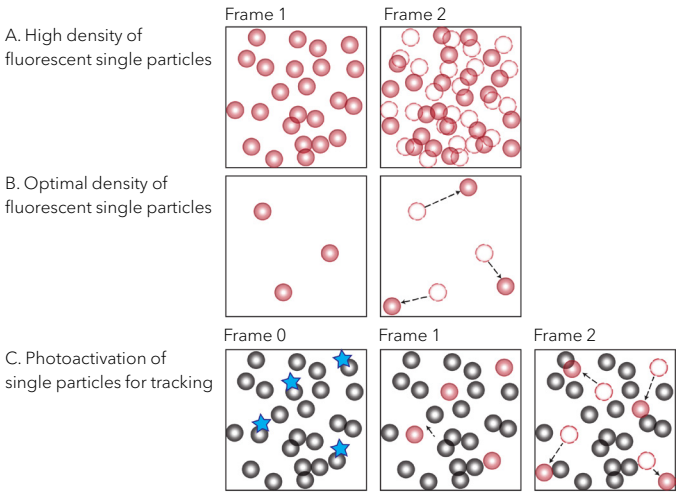

Figure 1. The concentration of labeled particles is important for determining particle trajectories. (A) A high density of fluorescent particles results in inaccurate tracks due to overcrowding and erroneous linking of each particle's position from frame to frame. (B) An optimal density of fluorescent single particles is characterized by single particles that are spaced sufficiently apart so that the position of each particle can be accurately recorded from one frame to the next without other particles occupying the nearby space of the tracked particle. (C) When the concentration of particles is high, photoactivatable fluorophores can be used to activate the fluorescence of a subset of labeled particles using UV light followed by tracking the photoactivated fluorescent particles.

4. Sample preparation and labeling

In vitro labeling

The labeling process is relatively simple and easy to perform by following the manufacturer's protocol. One tip our team shares is to coat the dish with 2M Glycine if you find that dyes are sticking to the glass. Use about 100 nM JF549, which is much less than for instance what Promega recommends, and prewarm and equilibrate the media in the incubator for all your washes.

Labeling of living cells - CRISPR/Cas9

HaloTags should be introduced at the endogenous locus by genome engineering. There are exceptions, such as primary cells where you may have to express the fusion protein from a virus for example. It is important to remember that the dynamics of proteins are usually defined by their abundance relative to their binding sites or partners so overexpression will skew this balance and may give you misleading results.

For immortalized mammalian cells introducing the tag is straightforward using CRISPR/Cas-9. A mix of Cas9, gene specific guide RNA and a repair template including your tag and sequences flanking the insert site are transfected into the cells and after several weeks clones are screened for HaloTag insertion.

One of the biggest obstacles with CRISPR is screening and identifying homozygous clones where all copies of the gene of interest have the correct modification, especially as many cell lines have three or more copies of genes.

Tip! Make use of the fact that the HaloTag can be fluorescently labeled and isolate the edited cells using fluorescence activated cell sorting (FACS). This will reduce time wasted in screening clones, when the CRISPR has failed and it is also possible to see multiple populations representing your wild type, heterozygous and homozygous cells. Then, it is easier to sort the homozygous cells guaranteeing something to work with after clonal isolation.

Cas9 plasmids from Feng Zhang's lab are available on Addgene and there are free websites to help you select your guide RNA. For most immortalized cell lines, the whole process takes about 4-6 weeks and you can do multiple cell lines in parallel.

Preparation for imaging

- Start the Nanoimager and enable temperature control to 37°C (usually takes ~60 min).
- Pre-warm the same culture medium used for culture and staining solution. Use a water/dry bath at 37°C to warm up the medium and staining solution.
- Aspirate the supernatant, and wash the cells thrice with prewarmed medium. Each well of 8 well Ibidi would require at least 200 µL of solution.
- Add the pre-warmed staining solution and incubate for 1 minute at 37°C.
- Wash three times with prewarmed medium.
- Add 200 µL of prewarmed medium after the final wash and proceed with imaging.
- Adjustment to the concentration of the staining solution might be required depending on the cell type and time of incubation.

It is also important to consider controls, such as HaloTag alone, or something stable like NLS-HaloTag or tagging of a histone.

5. How to perform imaging for SPT

During imaging it is important to consider different settings, including: laser power, exposure time, and duration of the event to track. To perform single particle tracking we acquire a movie of a fluorescently labeled protein so that only a small fraction are active at any one time.

Important considerations

- Once the sample is labeled it is best to get it onto the microscope quickly as the cell will start synthesizing unlabelled protein.
- The imaging medium should be the same culture medium but without phenol-red as this is mildly fluorescent. If you are imaging without a CO2 supply then adding 20 mM extra HEPES to the media will help with buffering.
- Once the sample is on the microscope you have about an hour to image before the cells start getting unhappy due to phototoxicity.
- For SPT, it is recommended to image in HiLO mode, which means the light is coming in at an angle. This improves the signal to noise and reduces the bleaching of the out of focus molecules.
- Exposure time should be 20-30 msec. Longer exposure times can lead to the fluorophores starting to blur.
- Once the exposure time is set you should play around with the laser power to find a setting with optimal spot density.
- We recommend imaging any one spot for about 5 minutes before the cell becomes unhappy.

In the illustration below, we start off with only inactive molecules and in the second frame one becomes active. We localize the x and y coordinates of the spot in each frame and if two spots are close together in subsequent frames, they are linked together. By acquiring a movie with high frame rate we can resolve tracks for hundreds of milliseconds until the molecule bleaches or diffuses out of focus. Over a five minute movie, thousands of tracks can be recorded in a single cell, allowing to perform population statistics.

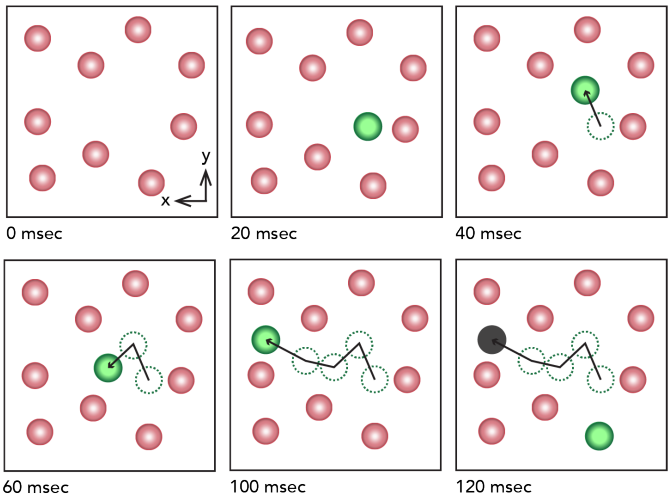

Figure 2. Cohesin-Halo-JF549 in U2OS cells.

6. Analysis of SPT data

There are a number of tracking programs that will localize the spots and make the tracks.

- The Nanoimager software (NimOS) has the ability to perform these functions.
- Trackmate is a popular tool used for researchers; spot-on takes the analysis of those tracks to the next level, providing stats and plots that can go directly into publication figures.

In this example, using data acquired on the Nanoimager, we can track the proteins and generate plots like this one where the tracks are coloured by diffusion coefficient with magenta being the slowest and cyan being the fastest. From these results we can see the spatial distribution of fast and slow moving molecules and interestingly that there are gaps where the molecules do not diffuse through.

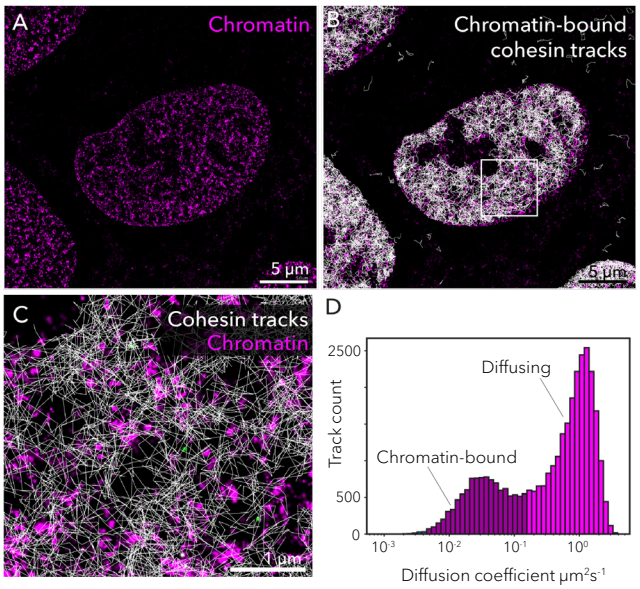

Figure 3. SPT of cohesin on chromatin in U2OS cells. (A-B) Maximum projection image at time 0 of U20S cells expressing Dy505-SNAP-H3 (chromatin) and SCC1/Rad21-HaloTag labeled with JF549 Halo ligand. Cohesin tracks shown in white were generated from time lapse images in NimOS. (C) Magnified view of the inset in (B). (D) Graph shows the binned distribution of diffusion coefficients in the chromatin-bound and the diffusing populations, and number tracks in each bin.

References

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3. Knight SC, Xie L et al. Dynamics of CRISPR-Cas9 genome interrogation in living cells. Science. 2015; 350 (6262): 823-6. doi: 10.1126/science.aac6572