Predictive Epigenetics: Fusing Theory and Experiment

Title of the protocol: Single-Molecule Tracking with HaloTag

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Introduction:
Live-cell single-molecule experiments are increasingly used to study the dynamics of biological processes such as gene regulation, signal transduction and self-assembly. The last decade has seen a lot of innovation to work around the limitations of low photon count, photo stability and low signal-to-noise ration.
The Halotag is a genetically encoded, 33kDa protein that becomes fluorescent when conjugated with a ligand. It retains the advantage of genetic encoding of fluorescent proteins, while profiting from the good photophysics of organic fluorophores. The decoupling of protein expression and its fluorescent also provides additional control to the experimenter. It allows for sparse labeling of the protein population, which is crucial for accurate localization of the molecules and their subsequent tracking.
Genetic encoding means that the protocol depends on having a functional HaloTag fusion protein expressed within the cell. Whether one chooses knock-in protocols, or an expression vector is up to the experimenter and the aims of the experiment. Nevertheless, this point must be considered in advance and the appropriate steps taken so that the cells studied are expressing the HaloTag protein at the time of labeling.

Description of the protocol:

Materials
- 4-well Lab-Tek
- growth medium
- optical medium
- ligand (JF549, or TMR, or any other suited to your system)
- PBS
- Hoechst 33342

HaloTag labelling for SMT
1. Plate the cells inside a 4-well Lab-Tek one or two days before the experiment (consider the possible requirements of the eventual transfection).
2. Prepare 550 µL of labeling solution per well by mixing cell medium and the ligand at the appropriate concentration (JF549 recommended range: 100pM-1nM)
3. Change the cell medium to the labeling solution. Incubate for 30 min at 37°C.
4. Wash gently with PBS, 3 times. Add fresh medium and incubate for 5 min at 37°C.
5. Prepare 550 µL of Hoechst solution by mixing cell medium and Hoechst 33342 at 1µg/mL.
6. Change the cell medium to the Hoechst solution. Incubate for 10 min at 37°C.

7. Wash gently with PBS, 3 times. Add fresh medium and incubate for 15 min at 37°C.
8. Change medium to optical medium and start the imaging.

Imaging

Guidelines
Acquisition parameters should be calibrated to the needs of your experiments. This includes laser power, exposition time, interval time between exposition, length of acquisition… For example, using a long exposition time, it is possible to blur out moving particle and focus on the bound ones. If time resolution is important – for measuring precise binding kinetics for example – consider shorter exposures, and higher laser power.
1. Select the cells to image based on the channel of the microscope for Hoechst, as to not introduce experimental bias by choosing the cells looking best on the ligand channel.
2. Capture a reference image of the DNA before switching to the ligand channel.
3. Expose the proteins to illumination to bleach them. Stop bleaching when the average amount of proteins visible simultaneously is around 10, and they are reasonably far apart.
4. Acquire the movie of the single-molecules.

Analysis
Many pipelines for analysis exist, most of them specific to each lab, as such we present this one as an indication. Tools have been developed on multiple platforms, and the particulars of the dynamics in which the experimenter is interested in might steer him towards some over others. The most popular solutions for this are DiaTrack, ilastik and TrackMate. We use the latter.

Guidelines
- Start by drawing the Region of Interest around the nucleus based on the DNA reference image.
- In TrackMate, for the particle localization step, the two parameters of interest of maximum spot size and the threshold. The maximum spot size can be inferred from the size of the protein, but visual check is necessary to determine parameter that work best. Use the preview tool at different time frames, possibly in different acquired movie, until false positives are eliminated (precision is preferable to sensitivity).
- For the linking step, one key parameter is the maximum displacement allowed between frames. It helps to have some idea of the expected diffusion coefficient for this, as one can derive the probability of a given displacement. This parameter is key, and we recommend running the algorithm and inspecting the results for any spurious tracks.
- Once working parameters are found, one can write a macro to fill in automatically the user prompts, so that the same parameters are correctly – and quickly – entered for each movie.
- TrackMate outputs a list of the tracks, composed of series of time-stamped position in the image. Further analysis of this data includes computing the diffusion coefficient D, the bound fraction, the kinetic parameters of binding molecules and more.

References:
Yu, J (2016) Single-Molecule Studies in Live Cells. *Annual Review of Physical Chemistry*, 67:565-85. Pubmed
Tinevez, J.Y., et al. (2017). TrackMate: An open and extensible platform for single-particle tracking. *Methods* 115:80-90. Pubmed