Single-molecule FRET on its way to structural biology in live cells

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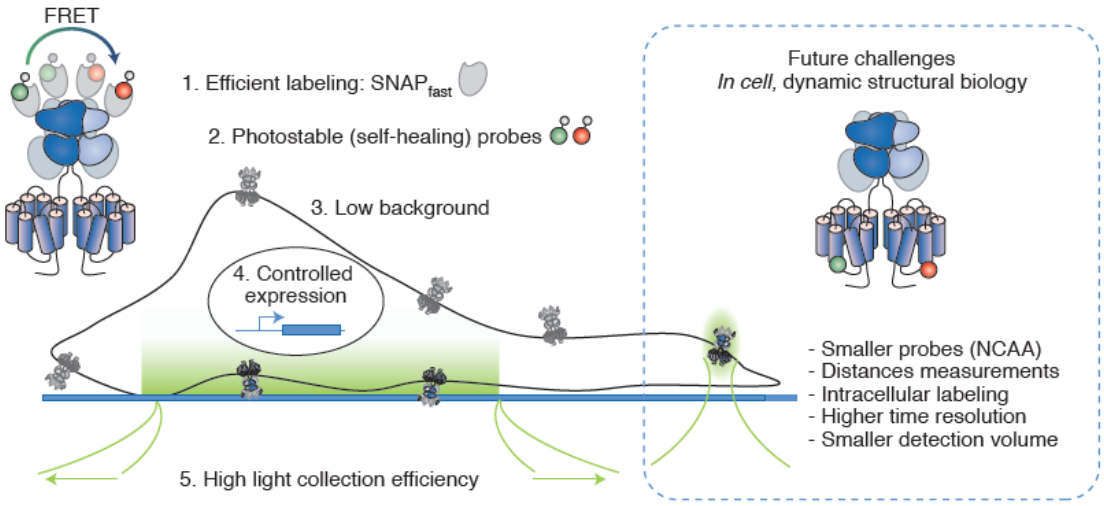

Figure : Toward distance and dynamic measurements in living cells using smFRET. Asher et al.[1] achieved the observation of conformational changes during the activation of expressed metabotropic glutamate receptors in living cells. Their strategy involved controlled expression, efficient labeling with cell impermeant and photostable probes, and improved detection. Further implementation of strategies established for superresolution microscopy or *in vitro* smFRET will soon lead to quantitative protein structural dynamics measurements in the context of living cells.

One of great challenges at the frontier of molecular and cellular biology is to obtain quantitative structural and dynamic information on macromolecular complexes in the context of living cells. Single molecule Förster Resonance Energy Transfer (smFRET) theoretically offers these possibilities, as it allows accurate distance measurements, refines biomolecular structures, and quantifies molecular dynamics[2]. However, obtaining such quantitative measurements in living cells offers many technological hurdles.

In this issue, Asher et al.[1] combined several cutting-edge approaches to monitor the assembly and structural dynamics of several G-protein Coupled Receptors (GPCR) using smFRET in living cells.

So far, smFRET measurements in living cells have mainly relied on the introduction of extrinsically labeled and purified proteins, therefore maintaining the concentration of the protein of interest at a concentration low enough for single molecule observation and circumventing non-specific labeling[3]. The approach used by Asher et al. focuses on strongly limiting GPCR expression via a weak, engineered and controllable promoter regulating expression from a single gene stably integrated into the expression host genome. Moreover, very efficient and specific double labeling of the receptors was achieved using SNAP$_{fast}$, a new generation of the well-established SNAP self-labeling tag with much faster labeling kinetics.

A second hurdle concerns the photostability of the fluorescent probes that is particularly crucial for smFRET measurements, where donor and acceptor fluorophores have to be “on” simultaneously. To limit the photo-destruction of the fluorophores *in vitro*, researchers usually rely on the use of

oxidizing/reducing chemical reagents and removal of oxygen from the solution[4]. Such harsh conditions can evidently not be used in living cells without impact on their viability. Fortunately, several groups have recently described the synthesis of "self-healing" probes that show much better photostability in common biological buffer conditions compared to classical probes[5]. These probes, modified here to react covalently with the SNAP-tag[1], turned out to be cell impermeant, which constitutes an advantage to increase the signal-to-background of the measurements on the cell surface. Indeed, they can be readily washed away and will not create a diffuse background within the cell.

Finally, the signal-to-background ratio for single molecule imaging was further improved by using total internal reflection microscopy (TIRF) with a very high numerical aperture objective lens (NA=1.7). TIRF illumination restricts the excitation of the fluorescent probes to a few hundreds of nanometers above the coverslip (*Figure*). Background fluorescence from the interior of the cell is therefore limited, and the diffusion of the receptors in-and-out of the illuminated basal membrane allows a steady flow of molecules to enter the observation area, ensuring proper statistics for the smFRET measurements. Here, the signal-to-background was further enhanced by taking advantage of the very high photon collection efficiency of the 1.7 NA objective lens. The drawback is that it requires the use of specialized and expensive coverslips and immersion oil, as compared to classical (1.45-1.49 NA) lenses used for TIRF microscopy.

Each of the optimization steps described above constitute incremental improvements. But their combination led to a new landmark in the field of smFRET in living cells, leading to the first observation of the structural dynamics of a membrane protein (the metabotropic glutamate receptor 2 (mGluR2))[1]. Nevertheless, obtaining live-cell quantitative structural information at the atomic level and at the timescale relevant for protein domain movements will require further improvements, as shown in the figure. This goal has been achieved for smFRET *in vitro*, combining innovations that are now mature for live-cell applications in the fields of protein engineering, illumination and detection technologies, and data analysis[2,6].

First, precise distance measurements require the use of smaller tags. Most of the self-labeling tags including the SNAP-tag suffer from their large size (a few nm), similar to that of fluorescent proteins, which also display poor photophysical properties for smFRET. A promising approach relies on genetic code expansion using uniquely reactive non-canonical amino acids. Proteins can be site-specifically equipped with FRET pairs through fast and bioorthogonal chemistries[7]. In combination with fluorogenic dyes that experience a "turn-on" only upon reaction, background can be minimized as has already been shown in superresolution microscopy studies[8], now opening up the way for efficient labeling within the cellular context.

Second, the proper time-resolution has to be reached. TIRF illumination combined with camera-based detection typically has a >20ms time resolution, which is too slow to monitor most protein domain conformational changes. smFRET observation in confocal geometry greatly improves the accessible time resolution down to the micro- or even nano-seconds. It offers access to the excited state lifetime of the fluorophores, and allows application of data analysis algorithms based on photon statistics that report and quantify fast protein motions[2]. Moreover, the confocal geometry is well suited for the observation of molecules at the apical membrane or even within the interior of the cells, and its throughput can be improved using matricial photodetector arrays[2].

Third, distance measurements between complexes of low affinity remain challenging. If the donor and acceptor are on two different biomolecules (*inter*-molecular FRET), the high dilution required for single molecule observation might lead to a dissociation of the complex. Here, the solutions may be related to those implemented in super-resolution optical microscopies. Reducing the observation volume is

one option, as protein concentration can remain high, while the single-molecule condition is maintained. This can be achieved for example using point spread function engineering (such as in STED microscopy for example[9]) or using optical nanoantennas, that restrict the illumination while increasing the photon flux, which bears additional potential to address fast structural dynamics below the microsecond timescale. Another option is to use photo-switchable dyes to limit the number of visible molecules in a given observation volume.

Community-wide efforts have been undertaken to standardize, benchmark, and validate smFRET as an *in vitro* platform to perform protein structural biology with angstrom precision and sub-µs resolution[6,10]. As briefly presented here, the creative combination and improvement of existing technologies will certainly lead to quantitative studies of protein conformational rearrangements in the complex environment of living cells, tissues or even multicellular organisms in the future.

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