IMAGING

# uPAINTing the plasma membrane

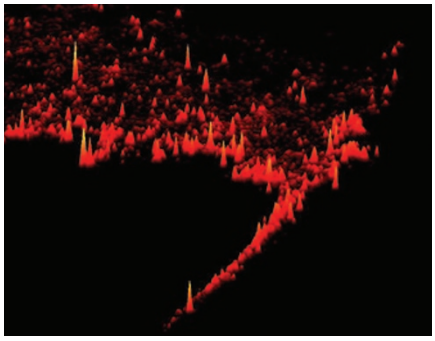
**Using real-time labeling with fluorescent ligands, researchers track thousands of endogenous proteins at the surface of living cells.**

The plasma membrane is a complex, dynamic structure in which countless molecular entities are constantly on the move, interacting with one another as well as with other molecules both within and outside the cell. How does one understand such a structure in molecular detail?

One way is to track single fluorescently labeled molecules at the cell surface. Molecules can be labeled either by fusion to fluorescent proteins or using ligands tagged with dyes or nanoparticles. Current approaches that permit large numbers of single-molecule trajectories to be gathered typically involve fusion of the protein of interest to a photoactivatable fluorescent reporter and exogenous expression of the fusion protein in cells. But this might lead to problems in some cases. Both high expression levels and reporter fusion could cause changes in the behavior of the protein being studied.

At the University of Bordeaux, Laurent Cognet and colleagues were looking for a way around this. They wanted to track endogenous proteins on the surface of living cells, and to do so for a large enough number of molecules that they could statistically analyze the behavior of proteins on a single cell. "We wanted to test whether looking at a big ensemble of overexpressed molecules is very different from looking at a big ensemble of endogenous proteins," explains Cognet.

Instead of relying on selective photoactivation of a reporter fused to the protein of interest, the researchers used real-time labeling of surface proteins with fluorescent dye–labeled ligands. Not only does this solve potential problems of reporter fusion, it also allows molecules to be tracked for longer because of the relative stability of organic fluorophores. "We try to avoid autofluorescent proteins so that we can really have very long trajectories," says Cognet. "Most of the time autofluorescent proteins are not that stable because they have this great ability to switch between different states."



Three-dimensional rendering of a uPAINT image of a model transmembrane protein at the cell surface. Image courtesy of Laurent Cognet.

In their approach, named uPAINT, the labeled probe diffuses in solution and binds continuously to its target protein on the surface of living cells. The researchers use oblique illumination so that only fluorescent probes close to the cell surface are excited and imaged. Using uPAINT, Cognet and colleagues followed trajectories of endogenous membrane proteins for up to tens of seconds, in the best cases, and acquired data from thousands of molecules in a single cell. They could thus compare subcellular differences in diffusion behavior for a variety of model proteins, as well as for endogenous AMPA receptors in hippocampal neurons.

They obtained the longest trajectories with ligands that had multiple bright Atto-647N molecules, but Cognet emphasizes that the approach may use other dyes as well. "As long as you can see a single molecule, you can use any dye you want," he says. And as for all techniques that involve binding of a labeled molecule to a target, uPAINT is entirely dependent on a good ligand, one that binds tightly to its target. "Since you are doing real-time labeling and you don't rinse," says Cognet, "your ligand has to be very good. Apart from that, the method is absolutely straightforward."

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RESEARCH PAPERS

Giannone G. *et al.* Dynamic superresolution imaging of endogenous proteins on living cells at ultra-high density. *Biophys. J.* **99**, 1303–1310 (2010).