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SPOTLIGHT

Ultra high-speed single-molecule fluorescence imaging

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In two articles in this issue, Fujiwara et al. developed an ultrasensitive high-speed camera capable of single-molecule fluorescence imaging at a microsecond timescale (2023. *J. Cell Biol.* https://doi.org/10.1083/jcb.202110160). This major leap in detection speed enables the organization of plasma membrane and integrin-based adhesions to be probed in unprecedented detail (2023. *J. Cell Biol.* https://doi.org/10.1083/jcb.202110162).

The visualization of single-molecule dynamics is a powerful approach to decipher how the molecular building blocks of life—proteins, lipids, and nucleic acids—perform their functions (1). While fluorescence imaging has become a common tool in single-molecule analysis because of the molecular specificity, versatility, and live cell compatibility of fluorescent labels, there are major technical barriers in terms of the spatial and temporal resolution that can be achieved. Much of recent effort has focused on enhancing the spatial resolution of fluorescence microscopy—even approaching the Angstrom level in a recent study (2). In contrast, there remains a significant gap in high temporal resolution analysis. Due to thermal fluctuations, individual biomolecules in plasma membranes or the cytosol can be displaced by tens of nanometers—multiple times the size of the molecules themselves—within sub-millisecond timescales. Thus, nanometer spatial resolution should be matched with sub-millisecond temporal resolution to furnish a complete picture of single-molecule dynamics. For studies of the plasma membrane, such spatiotemporal limitation is particularly salient. Plasma membranes are closely associated with subjacent cytoskeletal networks, which exert profound influence on the mobility of lipids and membrane proteins as conceptualized by the picket fence model (3). Accordingly, single-molecule trajectories within the plasma membrane are expected to exhibit a wide variety of mobility characteristics such as "hop-diffusion," in which local motion within compartments is unhindered but long-range motion is impeded through interactions with either the transmembrane protein "picket" or the membrane cytoskeleton "fence." However, such dynamics have been challenging to observe, especially by fluorescence-based approaches, due to their intrinsic sub-millisecond timescales (4). To address this, Kusumi and colleagues developed an ultra high-speed camera capable of single-molecule fluorescence imaging at microsecond intervals (5). In addition to providing a significant speed boost to single-molecule imaging as well as super-resolution microscopy, this enabled them to beautifully demonstrate hop-diffusion dynamics in live cell plasma membrane, as well as to probe the nanoscale structure and dynamics in integrin-based adhesion complexes (6).

Many of the recent imaging techniques in cell biology are made possible by advances in camera technology. The availability of electron multiplying charge-coupled device (EMCCD) camera in the early 2000s made the observation of single fluorophores broadly accessible to biologists and soon afterward paved the way for wide adoption of single-molecule based super-resolution microscopy (7). Broadly speaking, camera performance depends on trade-offs between sensitivity, speed, and noise. While the design of the EMCCD camera traditionally provides lower noise, its read-out bandwidths are inherently more constrained. The complementary metal oxide semiconductor (CMOS) architecture offers an intrinsic speed advantage but was initially far noisier (8). Thanks to continual development, CMOS sensors began to surpass the overall performance of CCD sensors by the early 2010s. Indeed, light sheet and other high-speed fluorescence microscopy modalities become practical largely due to the related scientific CMOS (sCMOS) design, which achieves low noise and high dynamic range at the cost of speed. Very high-speed imaging is nowadays possible with CMOS cameras optimized for frame rate in the millions per second. These cameras have higher noise levels, however, and are typically intended to be used with ample illumination. Taking advantage of this blazing speed advantage, in the first article, Kusumi and colleagues addressed the noise limitation by devising an efficient scheme to amplify weak single-molecule

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fluorescence signals to a detectable level (5). They used an image intensifier to generate multiple photons for each incoming fluorescence photon—these are then conveyed to the CMOS sensor by high-efficiency optical-fiber bundles. Altogether, this accomplished more than an order of magnitude increase in speed (12.5X–56.3X) at the cost of sensitivity reduction by a factor of ∼2.4 compared with recent sCMOS cameras.

In high-speed single-molecule imaging, high excitation intensity is necessary to drive more fluorescence emission, essential for determining each single-molecule coordinate with high spatial precision. However, this comes at a cost of increased photobleaching, which affects the longevity of single-molecule trajectories. Thus, the speed limit for single-molecule imaging is dictated by the performance of the fluorophores rather than the camera itself. The authors identified Cy3 as offering optimal high-speed imaging performance in their system, capable of yielding lengthy trajectories with 20-nm precision at 100-µs intervals, and thus sufficient to monitor sub-millisecond plasma membrane dynamics. Previously, to achieve such high speed required the authors to use bright field microscopy and gold nanoparticles as labels (9), which is sub-optimal since the 40-nm gold probes are orders of magnitude larger than typical lipid molecule or transmembrane proteins, in addition to being sterically excluded from the basal plasma membrane.

Using Cy3 to label a phospholipid (DOPE) or transmembrane proteins such as transferrin receptor (TfR), the authors performed high-speed observation (6–10 kHz frame rate) and showed clearly that both phospholipid and TfR undergo hop-diffusion, defined as short-term (ms) confined diffusion within a compartment plus occasional hop movements to an adjacent compartment (5). Such hop-diffusion characteristics are uniquely dependent on high-speed observations—the confined diffusion within each compartment occurred for only a few to tens of ms and thus will be smeared out when observed at slower speed (e.g., at the conventional video rate of 30 Hz). The authors further corroborated the role of the actin cytoskeleton as the "fence" that compartmentalizes the plasma membrane by demonstrating that in the bleb plasma membrane, which is devoid of actin, both DOPE and TfR exhibited random Brownian motion instead.

To quantitatively address the composite nature of the single-molecule trajectories, the authors also developed a theoretical framework to estimate the dwell lifetime of each molecule in the membrane compartment. This enabled them to estimate the compartment sizes (∼109 nm) and inter-compartment hop frequencies, determining these to be ∼10 ms for DOPE and ∼24 ms for TfR. Remarkably similar values were obtained for both the apical and basal plasma membranes, indicative of their similar physical organization. Notably, in this study, a relatively simple cell model (T24 bladder carcinoma cell line) was used, and thus the similarity between the apical and basal membranes is perhaps to be expected. Nevertheless, these measurements now stand as the baseline against which spatiotemporal dynamics in more complex and specialized membrane context can be compared, whether in different domains of the apicobasally polarized epithelial cells or different regions of neuronal cells, to name a few examples. Another interesting direction for future investigation would be to probe how the spatial architecture and temporal dynamics of membrane cytoskeleton contribute to hop-diffusion: Are hop diffusion trajectories correlated with the actual "fence" geometry? Here, cells with relatively sparse actin cortex, such as embryonic stem cells, could serve as a tractable model system (10).

The ultrafast single-molecule imaging capability developed here also naturally lends itself to single-molecule-localization super-resolution microscopy. This is demonstrated in the companion article (6), whereby the authors demonstrated live cell photoactivated localization microscopy (PALM) using the mEos3.2 fluorescent protein with 1 ms exposure time per frame—more than an order of magnitude improvement in speed over previous studies. Using various super-resolution microscopy modalities together with single-molecule tracking, the authors then probed how key proteins within integrin-based focal adhesions (FAs) are organized at the nanoscale. As reviewed recently (11), earlier studies by the authors as well as other groups have shown that at the super-resolution scale, FAs consist of nanocluster aggregates or "islands" that readily undergo dynamic exchange, belying their monolithic appearance at lower imaging resolution. Here, the permeability of FAs is corroborated from the analysis of single TfR or integrins traversing the FAs. These also exhibited hop-diffusion characteristics, but with a smaller membrane compartment size of ∼70 nm for TfR, as expected. The authors also performed stoichiometric analysis of paxillin, a ubiquitous FA marker, using paxillin-null mouse embryonic fibroblasts to ensure no interference in molecule-counting from endogenous paxillin. This led to the estimate that typical FA "islands" may contain as few as six molecules of paxillin. Next, using two-color super-resolution analysis, they examined how core FA proteins such as integrin β1, integrin β3, talin, vinculin, and focal adhesion kinase (FAK) are co-distributed with paxillin islands. Their analysis yielded a complex picture of how FA proteins are organized. These FA proteins seem to form "island"-like nanoclusters with comparable mean diameters in the 30-nm range. Direct overlapping of "islands" of different FA proteins appears to be uncommon, similar to what has been observed by other groups and further highlighting the heterogeneity of FAs at the nanoscale (11). Instead, they found that the "islands" appear to be loosely organized into spatial domains with ∼300 nm correlation lengths. At present, the molecular origin of this multi-hierarchical organization is unclear. Such long-range inter-cluster coordination has been hypothesized to arise from either engagement with the actin cytoskeleton or large scaffolding proteins such as talin (11). As it is challenging to infer the physical relationship between these nanoclusters by fluorescence-based approaches alone, future studies to address this could perhaps benefit from multiplexing single-molecule techniques with a nanopatterning approach to introduce spatial constraints to FAs (12).

In summary, the authors presented here landmark observations on hop diffusion in live cell membranes. Their impressive technical advances discussed here should help open up a fresh spatiotemporal regime of membrane organization for further exploration, which is expected to be richly detailed, context-specific, and closely coupled to biological activity (13). Additionally, in terms of ultra high-speed, high-resolution single-molecule tracking, the

camera-based strategy described here offers a large field-of-view approach that should complement the sequential structured illumination methods such as MINFLUX, which is parametrized for high spatiotemporal tracking of relatively few copies of molecules (14). More broadly, the enhancement in sensitivity and speed will prove useful beyond single-molecule fluorescence imaging, augmenting the performance of techniques such as light sheet microscopy, for example. Finally, as there is still significant headroom in terms of raw hardware performance of this camera system, we could anticipate further improvement in imaging capability with the next generation of fluorophores (15).

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