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Caught in the Act: Quantifying Protein Behavior in Living Cells

Diane S. Lidke and **Bridget S. Wilson**
Department of Pathology and Cancer Center, University of New Mexico Health Sciences Center, Albuquerque, NM 87131

Abstract

Protein localization and dynamics both play an important role in cell signal transduction. Although biochemical studies have elucidated many details about the chain of events in signal cascades, the poor temporal resolution and absence of spatial localization in these conventional techniques make it difficult to determine the "where and when" of protein interactions. Over the past decade, imaging technologies and biological tools have developed to a point where many fundamental questions about protein activities can now be addressed at the molecular level in living cells, revealing spatio-temporal information that is not provided by traditional biochemical assays. In this review, we illustrate the power of emerging fluorescence microscopy techniques to capture and quantify protein dynamics.

Spatio-temporal Aspects of Protein Behavior

Complex cellular processes are governed by signal transduction, which in turn is controlled by protein-protein interactions at the plasma membrane and along the signaling cascade. While biochemical techniques have been used for decades to determine the order of protein interactions along signaling pathways, a current thrust in cell biology is to understand the role of protein dynamics in signal transduction. For example, a membrane receptor tyrosine kinase is in constant motion as it diffuses on the plasma membrane, interacting with other proteins and membrane microdomains. Ligand binding may lead to a change in diffusive behavior and is often the trigger for receptor homo- or hetero-oligomerization, which initiates receptor phosphorylation and cytoplasmic adaptor protein recruitment. The final step is eventual removal of the activated receptor from the plasma membrane via endocytosis, after which the receptor may be recycled or continue along the endosomal pathway to degradation. Despite this common picture of protein events, there are many details in this sequence that remain unclear. The low spatial and temporal resolution of traditional biochemical techniques cannot reveal the spatial distribution and dynamic aspects of these processes. Recent developments in fluorescence microscopy are allowing us to probe protein behavior in real-time, such that we can directly visualize and quantify signal transduction events within the living cell.

Classic techniques for identifying protein-protein interactions

Evidence for direct protein interactions is often first established using biochemical methods (immunoprecipitation, pull-down assays) or genetic approaches (2-hybrid and complementation assays). To confirm the existence of protein complexes in cells, cell

Corresponding author: Lidke, D.S. (dlidke@salud.unm.edu).

biologists often rely on conventional fluorescence imaging techniques for detecting colocalization or engineer pairs of fluorescent proteins for more sophisticated FRET analysis. However, these methods have their shortcomings. Traditional colocalization of two or more fluorophores in wide-field or confocal fluorescence imaging demonstrates whether labeled proteins are in the same general sub-cellular location, but cannot directly detect complex formation because resolution is limited to >250 nm. FRET (Box 1) can reliably report protein interactions[1], since proteins must be within 1–10 nm of each other of each other for energy transfer to occur, but suffers from complications that can lead to false negative results. For example, the distance between labeled components in large multi-protein complexes may be too far for FRET to occur. Therefore, a lack of energy transfer is not necessarily evidence for a lack of protein complex formation. Typically, FRET is limited to the measurement of interactions between two protein species, but creative approaches can expand this capability. For example, Grant and colleagues recently introduced four different fluorescent species into the same cell to permit simultaneous FRET measurements of two distinct FRET pairs[2]. Two-color cross-correlation FCS (Box 1) is a valuable alternative method for detecting protein interactions, with the limitations that this technique provides measurements only at a fixed location and is suitable over a narrow range of protein concentrations. Recent advances in fluorescence microscopy techniques (Box 1), such as image correlation microscopy, super-resolution techniques and FRET imaging, are providing new ways to address questions in cell biology. These creative approaches measure the average behavior of an ensemble of proteins and are developing at a fast pace.

Another classic technique to probe protein dynamics in cells is single particle tracking (SPT; Box 1). SPT reveals behavior at the molecular level by tracking the motion of individual proteins, teasing out details not distinguished in methods that measure the average behavior of populations of molecules. Here, too, there have been significant recent technological advances. In the past, SPT relied on large, highly multivalent colloidal gold probes[3], which introduced complications associated with crosslinking of the molecule(s) being tracked, or relied on easily photobleached fluorochromes that limited tracking to very short time scales[4]. The introduction of improved fluorescent probes, such as the bright and photostable quantum dots (QDs; Box 2), greatly increases the duration of fluorescence-based SPT[4–6]. Another advantage of QDs is their broad excitation spectrum that allows for simultaneous excitation of many spectrally distinct QDs with a single wavelength. Combinations of multi-color probes and multi-spectral imaging allow for tracking of multiple protein species simultaneously, providing a reference frame for protein motion.

Emerging fluorescence microscopy techniques can quantify protein behavior in the living cell and thus enable new and important discoveries that could not have been gained through biochemical techniques. In this review, we highlight some examples of how advanced fluorescent microscopy techniques have provided new insight into longstanding biological questions. In particular, we focus on two main areas of cell biology study: the influence of membrane microdomains on protein behavior and the quantification of protein-protein interactions. We also describe some technologies that we predict will have an increasing impact in cell biology.

Domains and corrals restrict membrane protein movements

Understanding the influence of membrane architecture on protein function is a major theme in membrane biology[7–9]. SPT has provided evidence for nanometer-sized membrane domains that restrict the lateral diffusion of membrane constituents. Along with biochemical fractionation techniques, these observations contributed importantly to several alternative hypotheses of membrane microdomain organization, such as lipid rafts[10], protein islands [11] and actin corrals[12,13].

Elucidating the contributions of microdomains in restricting protein diffusion is needed to fully determine how these restrictions may limit receptors accessibility and govern signaling processes. For example, is membrane organization dominated by large scale segregations of proteins within the lipid sea (i.e., the protein island hypothesis)?[11] Alternatively, is membrane architecture primarily driven by phase separation of lipids and their associated proteins (i.e., the lipid raft hypothesis)?[10] How does the cortical cytoskeleton interact with proteins to create “confinement zones” (i.e., actin corrals or picket fence hypothesis where proteins collide with the cytoskeletal fence or transmembrane proteins bound to the cytoskeleton)?[14] Evidence from multicolor imaging and long-term SPT suggest that microdomains provide a plausible explanation to explain the restricted diffusion of proteins. Microscopy has also shown that this partitioning of proteins on the plasma membrane into confinement zones can be transient, where proteins transition from confined to free diffusion or between adjacent confinement zones (hop diffusion).

Diffusional trapping (Protein Islands)

In a paradigm-shifting study, Douglass and Vale used a combination of TIRF (total internal reflection fluorescence) microscopy, confocal imaging, FRAP and SPT to provide evidence that discrete microdomains in the T cell membrane transiently trap diffusing signaling proteins [15]. At the outset the authors observed that mRFP-tagged CD2, a non-raft transmembrane protein, was highly immobile. Moreover, the red fluorescent CD2 population could be imaged as large, relatively stable clusters at the adherent surface of T cells imaged in TIRF. In contrast, important signaling molecules such as the transmembrane adaptor LAT, the tyrosine phosphatase CD45 and the tyrosine kinase Lck displayed both highly mobile and immobile fractions. GFP fused to the amino terminal of Lck (Lck10-GFP), which results in dual acylation, served as a "lipid raft" marker. Contrary to the prediction that raft associated proteins would have a low diffusion coefficient, Lck10-GFP demonstrated both fast average diffusion and a very small immobile fraction. After acquiring a fixed image for CD2 fluorescence, the team switched to SPT mode to track GFP-tagged signaling proteins. By use of an overlay approach, they were able to evaluate the diffusional properties of single GFP-tagged proteins relative to the more stable CD2 regions. This provided the first concrete evidence for diffusional trapping, since both LAT and Lck were observed to have restricted mobility within CD2-defined microdomains. Abrupt changes in LAT and Lck mobility could often be seen, which correlated strongly with entry and exit from CD2 domains. This work was possible through creative use of multicolor imaging in SPT and ensemble modes, where the latter provided a reference frame for the individual molecules being tracked.

Ehrensperger and colleagues used similar SPT techniques to show that diffusional trapping occurs in neuronal cells, suggesting that plasma membranes of most cells harbor microdomains that restrict lateral movement of proteins[16]. In this study, motion of the neurotransmitter receptor, GlyR, was tracked as it traveled in and out of gephyrin clusters. While the gephyrin clusters were again marked with a chimeric gephyrin protein fused to the fluorescent protein Venus, the researchers chose to tag the GlyR with QDs. The use of QDs provided a higher signal/noise ratio and longer imaging times for tracking single GlyR molecules, compared to the GFP-tagged proteins in the Douglas and Vale study. This approach enabled the authors to extract the kinetic parameters that govern the equilibrium between GlyR exchanging in and out of gephryin clusters. Sophisticated mathematical analysis of QD-GlyR motion suggested that at least two subpopulation of receptors coexist within gephryin clusters and support novel hypotheses regarding multiple association states of protein complexes.

Actin corrals

By simultaneously tracking the dynamics of GFP-tagged actin and the trajectories of QD-labeled FcεRI, our group was the first to directly visualize actin “corralling” of membrane

proteins[5] (Figure 1). This was a particularly important aspect of the study since, unlike CD2 or gephyrin clusters, the actin cytoskeleton is highly dynamic and the structural network is markedly altered on the order of seconds. Data were acquired both in TIRF, to image events at the adherent surface of mast cells, and in confocal mode, to image receptors and actin at the top of the cell. The use of QDs (Box 2) was imperative to provide long trajectories (>30s) without photobleaching so that spatial proximity and diffusional behavior of receptors relative to actin reorganization could be readily captured. This work demonstrated that membrane proteins can be transiently trapped within an actin-defined region of the membrane. Furthermore, as the cytoskeleton rearranges, the receptors can slip through temporary openings and move to new regions. Mathematical analysis of FcεRI trajectories as they approach actin revealed that the receptor does not interact with actin. Instead, actin acts as a physical barrier to protein diffusion.

Lipid Rafts

The apparent small size and dynamic nature of lipid rafts have generally precluded their detection with light microscopy. However, recent results from Eggeling and colleagues using STED (Box 1) have provided convincing evidence for lipid raft involvement in membrane compartmentalization[17]. The enhanced lateral resolution of STED (down to ~40 nm) makes it possible to probe very small areas of the membrane – regions on the order predicted for lipid raft size - and is ideal to create a smaller focal volume for FCS analysis. Results showed that fluorescently-labeled GPI-anchor proteins, as well as sphingolipids, are briefly trapped in nanometer sized (<20 nm) domains that are cholesterol dependent. An important feature of this paper is the comparison of single molecule traces acquired by STED with those acquired by confocal imaging, demonstrating that only the STED approach could capture the heterogeneous diffusion of sphingomyelin.

Clustering and oligomerization are measurable processes

An important goal in cell biology is to monitor the ebb and flow of molecular complexes that tune the overall responses of the cell. For example, what are the lifetimes of protein complexes formed during signaling and where do these productive protein interactions occur? The techniques discussed so far are powerful in their ability to obtain dynamic information from single molecules and relate *individual protein* behavior to its environment. Techniques that measure the behavior of a *population of molecules* at once can also be used to determine the location and time scale of protein interactions. Such techniques include FRAP, FRET and fluorescence correlation methods (Box 1).

Protein Oligomerization

As previously introduced, FRET is one approach used to detect close proximity between proteins. This technique has been successfully applied by many researchers to determine dynamic changes in protein-protein interactions. In one recent example, Liou and colleagues combined information from traditional FRET and FRAP to examine the oligomerization and translocation of STIM1, an endoplasmic reticulum (ER) resident whose role is to detect depleted calcium stores and stimulate store-operated calcium entry[18]. After triggering release of Ca^{2+} from ER stores, the authors observed a marked increase in energy transfer between CFP- and YFP-STIM1, consistent with protein aggregation. Live cell imaging also revealed that STIM1 translocates within the ER to form clusters near the plasma membrane (PM). This ER to PM translocation was apparently limited to locally available STIM1, based upon the very slow recovery of YFP-STIM1 after photobleaching. Together, the data suggest that signaling is spatially localized to a subpopulation of STIM1 located within a region of ~2 μm near putative ER-PM junctions.

Robia and colleagues have recently modified the FRET approach to determine protein exchange within an oligomer[19]. Förster transfer recovery (FTR) is a clever combination of FRET and FRAP in which the acceptor is photobleached and the recovery of energy transfer (rather than just intensity) in the bleached region is monitored over time. From the kinetics of the energy transfer recovery, one can determine the exchange rate between bleached and unbleached molecules in a complex. The authors used this technique with YFP- and CFP-fusion proteins to determine that phospholamban exchanges quickly in regulatory complexes with the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) but forms relatively stable homo-oligomers.

In the case of homo-interactions, the need for labeling with two different fluorophores is not ideal. Since the same principles that govern FRET between chemically different donor and acceptor fluorophores also applies to energy transfer between like fluorophores (i.e. GFP to GFP), the application of homo-FRET to live cell imaging makes it possible to quantify homo-clusters using a single class of fluorescent tag[20,21]. Steady state and time-resolved fluorescence anisotropy imaging enables sensitive measurement of homo-FRET, based upon the rapid depolarization of the fluorescence. Importantly, homo-FRET can report the *number* of fluorophores in a complex or cluster. This approach was pioneered by Sharma *et al*, who focused on the clustering of GPI-anchor proteins (GPI-APs)[22]. This study showed that GPI-APs are found as both monomers and in nanoscale (<5nm), cholesterol-sensitive clusters on the plasma membrane. More recently, Bader *et al*[23] employed time-resolved anisotropy measurements in a specially equipped confocal microscope to spatially resolve differences in protein aggregation state across the cellular landscape (Figure 2). Using this instrument, they were able to distinguish that GPI-APs form small clusters on the plasma membrane, yet remain monomeric when residents of internal organelles.

Ligand-induced behavior

When used for multi-color imaging, SPT can also report protein-protein interactions[5,24, 25]. While in the Jovin group, Lidke directly conjugated EGF to QDs to facilitate tracking of ligand-bound EGFR/erbB1[26]. The bright, photostable QD probes made it possible to directly observe the process of receptor internalization and, by use of cells stably transfected with erbB2-YFP or erbB3-mCitrine, to quantify difference in co-internalization of heterodimers. In a subsequent study, the use of EGF-QDs permitted detection of rapid retrograde erbB1 transport down cellular filopodia prior to endocytosis[24]. By combining single QD tracking and FRAP of GFP-actin, retrograde transport was shown to be coupled to actin flow rate and not dependent on a motor protein. This study was amongst the first to exploit the capabilities of two-color single QD tracking, establishing the stability of erbB1 homodimers bound to EGF-QD525 and EGF-QD605 and demonstrating that dimerization was a prerequisite for retrograde transport.

Mapping protein diffusion and aggregation

Another rapidly developing family of techniques involves the use of fluorescence correlation methods. FCS was first used to measure binding interactions in solution[27]. Cell biologists quickly adapted this technique to measure protein diffusion and aggregation state in living cells. As examples, FCS methods have been used to determine the aggregation state of membrane proteins[28], interactions between membrane proteins and downstream signaling partners[29] and the stoichiometry of protein complexes[30]. An inherent limitation of FCS is that measurements are recorded at a single position in the cell, which is defined at the beginning of the experiment. Image correlation techniques overcome this limitation by providing mobility and aggregation state data along with spatial information, generating maps of protein dynamics and interactions across a cell[31]. Importantly, these new methods can be performed using standard confocal laser scanning or TIRF microscopes that are widely available in academic core facilities. Image correlation methods have been applied to a wide variety of biological problems, such as measuring actin-integrin interactions by velocity mapping[32] and

determining integrin aggregation state in adhesion organization[33]. Improvements in quantitation have been made, such as new analytical methods for calculating the fraction of interacting proteins from two-color ICCS data[34]. The Wiseman group makes available programs for analyzing data from many ICS techniques (http://wiseman-group.mcgill.ca/).

Recently, ccRICS and ccN&B analysis (Box 1) have emerged as promising new methods. Both are based on cross-correlating fluorescence in two-color pairs of images acquired by laser scanning confocal microscopy. Digman *et al* have used ccN&B to evaluate the exchange of focal adhesion kinase (FAK), paxillin and vinculin within adhesion complexes of fibroblasts [35]. Based upon the amplitude of the correlated fluctuations, it was possible to determine the stoichiometry of proteins in large aggregates that dissociated from adhesions as they disassembled. This group has also used ccRICS to create localized "brightness" maps, reflecting the dynamics of these proteins as they enter or leave adhesion structures[36]. As a concluding remark for this section, we note that attempts to capture the dynamics of FAK and vinculin binding to Pax by FRET methods were unsuccessful[36]. Clearly, the choice of analytical technique for measuring specific protein-protein interactions must sometimes be a trial and error process.

On the forefront

The examples described above demonstrate the ability of fluorescence microscopy to obtain information about biological processes that could not have been gained with conventional techniques. However, many other biological questions still exist that will require new technology, including: 1) While tracking multiple protein species simultaneously with multi-color imaging is useful, is there a way to see more than the typical 2 colors? 2) Can we track protein motion in three dimensions, permitting observations of proteins restricted by junctional complexes in polarized cells or moving through the interior of the cell? 3) Can we combine super-resolution with high temporal resolution to monitor dynamics at the nanometer scale? In the following sections, we describe applications of both established and new techniques that demonstrate these challenging questions *can* be addressed by the cell biologist.

How can we see more?

The ability to track the motion of specific proteins with respect to a reference (other proteins or lipids) using two-color imaging has already proven to provide much more information than single color imaging. In the quest to resolve more proteins, multiplex or multi-color imaging holds much promise. However, filter-based imaging systems are typically limited to 2–4 different fluorophores due to limitations in excitation sources and overlapping emission spectra of conventional dyes. New generation hyperspectral microscopes will greatly increase the number of labels that can be imaged simultaneously, such as the confocal imaging system developed by Sinclair *et al*. While several commercial hyperspectral microscopes are available (i.e. Zeiss META), this new instrument acquires the full emission spectrum at each sampled point with an exceptional 1–3 nanometer spectral resolution[37]. When combined with sophisticated analysis routines, even closely overlapping fluorophores can be distinguished [38]. Spectral imaging can also improve FRET measurements[39,40].

Tracking protein motion in 3D

Traditional imaging techniques are limited to a 2D focal plane. Several groups are developing 3D tracking systems based on different approaches[41–45]. Ober and colleagues have designed an instrument that allows for simultaneous imaging in two focal planes: one at the membrane surface by TIRF and the second inside the cytoplasm by epifluorescence. Using this microscope, they have monitored protein endocytosis, recycling and exocytosis in real-time [43,46]. Werner and colleagues have taken a different approach by developing a microscope

that “locks onto” a single QD probe and tracks its motion in x, y, and z by moving the microscope stage to always keep the QD in focus[42,47] (Figure 3). Manipulation of the excitation or emission light can also provide information in the z-dimension. In astigmatic imaging, a cylindrical lens is used to introduce an xy asymmetry in the fluorphore emission that is related to z-position[41]. This technique has recently been combined with STORM to generate super-resolution 3D images of the microtubule network[48] and in single QD tracking to study intracellular transport[49]. Pavani *et al* have demonstrated the ability to localize molecules in the z-dimension using a special shaped double-helix point spread function[50]. Hagen *et al* have recently developed a Programmable Array Microscope (PAM), that combines structured illumination and detection to produce video rate optical sectioning with photobleaching in arbitrary regions of interest, and used the PAM to measure diffusion of the membrane protein erbB3[51].

Capturing protein dynamics at super-resolution

Recently, a number of super-resolution imaging techniques have been developed that can “break” the diffraction limit of the light microscope and provide 100 nm or better resolution with light microscopy[52]. For example, new single molecule localization techniques are powerful ways to map protein localization, but initially relied on the sequential localization of individual fluorophores in fixed cells and required minutes to hours to generate super-resolution images. The field is now focused on bridging dynamic and super-resolution measurements through the use of novel, photoactivable probes in live cells. Techniques like sptPALM[53] and live-cell PALM[54], discussed in the article by Lippincott-Schwartz in this issue, are examples of this emerging technology. Live imaging with STED has also been performed to image dendrite spine or organelle motion with rates up to 1 frame every 10 s [55,56]. Structured Illumination Microscopy (SIM) is another super-resolution technique that can increase lateral resolution over conventional microscopes. In SIM a sample is illuminated with a series of patterned light and computational analysis reconstructs the super-resolution images from high frequency information encoded in Moiré fringes[57] and has recently captured kinesin and microtubule dynamics with 100 nm resolution[58].

Bright future for quantitative imaging

The fluorescence imaging techniques described here are capable of capturing biochemical and biophysical events in the living cell. These emerging techniques provide the opportunity to examine cellular events on unprecedented scales in time and space. With up-and-coming technologies poised for innovative biological applications, the cell biologist will be able to address questions that have previously been experimentally inaccessible. Importantly, many of these techniques can be performed using commercially available instrumentation. The trend is clear: fluorescence microscopy will play an increasingly important role in cell biology, shaping the way cell biologists approach questions and providing quantitative information that compliments and extends traditional biochemical techniques.

Box 1: Comparison of fluorescence microscopy techniques used to measure protein-protein interactions and dynamics

Here we compare and contrast some common fluorescent microscopy techniques that can monitor protein-protein interactions and dynamics. It is important to consider the advantages and limitations (Table I) of each, as well as the accessible spatio-temporal scales (Figure I), when deciding which technique is best suited to address a particular biological question.

Fluorescence Recovery After Photobleaching (FRAP)

Fluorophores in a small region of interest are photobleached with a short burst of intense laser excitation. As non-bleached molecules diffuse into the bleached region, fluorescence intensity is recovered and a diffusion constant can be calculated from the fluorescence recovery time.

Förster Resonance Energy Transfer (FRET)

Non-radiative transfer of energy from a lower wavelength (donor) to a higher wavelength chromophore (acceptor) that is dependent on the distance between them. This can be observed by a decrease in donor emission, a decrease in donor lifetime or an increase in the emission of the acceptor. FRET between like molecules can also be achieved (**homo-FRET**), with the added advantage of using a single fluorphore.

Single Particle Tracking (SPT)

Sparse labeling of proteins in cells allows for direct tracking of individual protein trajectories, which can be analyzed to determine diffusion constants and the type of motion (free, restricted, immobile).

Fluorescence Correlation Spectroscopy (FCS)

A small focal volume is defined with a focused laser beam and a confocal pinhole or by two-photon excitation. Intensity fluctuations are generated as fluorescently-tagged proteins diffuse in and out of the focal volume. Diffusion constant can be determined by fitting theoretical models to the autocorrelation of the intensity trace.

Image Correlation Spectroscopy (ICS)

Changes in fluorescence intensity across an image are used to calculate the spatial correlation function. Number density and aggregation state are determined by fitting models to the correlation function. Images can be acquired by confocal or TIRF microscopy. Variations of this technique include Temporal ICS (measures dynamics and number density) and Spatiotemporal ICS (returns velocity maps). These methods evaluate changes over time using confocal time series data.

Raster Image Correlation Spectroscopy (RICS)

RICS uses the time information inherent in a confocal image, created by the raster scanning of the laser across the field of view. This measures interactions on the order of μs-ms. Calculation of the correlation function using this time information can generate diffusion maps across the image.

Number and Brightness (N&B)

N&B is based upon pixel-by-pixel analysis of images in a confocal time series, with a focus on intensity fluctuations that reflect diffusion of molecules in and out of a pixel. It is possible to determine the brightness of an individual molecule and then estimate the number of molecules per pixel.

Cross-correlation (cc)

Variation on each of the above (FCCS, ICCS, ccRICS, ccN&B), based on two-color imaging. Quantification of coincident (spatial and/or temporal) fluctuations between the two channels provides information on fraction of proteins interacting, dynamics and stoichiometry of complexes.

Stimulated Emission Depletion (STED)

Combines a standard Gaussian excitation beam with a doughnut-shaped beam that depletes emission from the outside ring of the excitation spot, resulting in emission only from the central (~40 nm) region.

Localization microscopy

Intermittency in fluorescence (blinking, binding/unbinding, photoactivation/photoswitching) allows for isolated fluorophores to be localized one at a time, building up an image with ~10 nm localization accuracy. Examples include STORM[59], PALM[60], FPALM[61], and PAINT[62].

Box 2: Quantum Dots

There are a range of fluorescent labels that can be used for visualizing proteins, including organic dyes and fluorescent proteins[69]. Quantum Dots (QDs) are a relatively new fluorescent probe composed of a semi-conducting nanocrystal core surrounded by a passivation shell and a water-soluble polymer coating[70]. A detailed comparison between QDs and organic fluorophores has recently been presented by Resch-Genger[71]. Here we briefly outline the common advantages and disadvantages of QDs.

Advantages

Photostability

QDs are photostable over minutes and even hours[4]. One caveat is that, with high excitation power, QDs can photodegrade resulting in a blue-shift of emission and eventual loss of signal[72].

Brightness

The large extinction coefficient and high quantum yield of QDs results in high brightness.

Flexible conjugation schemes

A variety of QD conjugation schemes are possible and many different coatings for QD conjugation are commercially available[70].

Broad excitation spectrum

QDs can be excited in a continuum of wavelengths below their emission spectrum, with absorption increasing towards the UV. This makes it possible to simultaneously excite many spectrally distinct QDs (or QDs plus GFPs or other dyes) with the same excitation wavelength.

Narrow emission spectrum

Their narrow emission spectra facilitate filter-based separation of spectrally distinct QDs.

Multiple colors

QDs are available in a spectrum of emission profiles.

Electron dense

The nanocrystal core can be imaged by electron microscopy[73].

Disadvantages*

Size

Typically 10–20 nm in diameter, much larger than an organic fluorophore, may cause steric interference with protein function.

Multi-valent

The multi-valency of QDs can be a complication when monovalent labeling is required. Methods have been developed to optimize 1:1 stoichiometry[74,75].

Blinking

Intermittency of emission is a property of most QDs. This can cause complications in single QD tracking, such that when the QD is “off” the molecule can be lost. To minimize this complication, several single QD tracking algorithms have been developed[4,5,16]. Additionally, two groups have reported synthesis of non-blinking QDs[76,77].

*These perceived disadvantages of QDs have been exploited by researches in particular experimental designs. For example, the blinking of QDs can be used for super-resolution imaging[78] and the multi-valency can be useful in situations where crosslinking of proteins is required[79].

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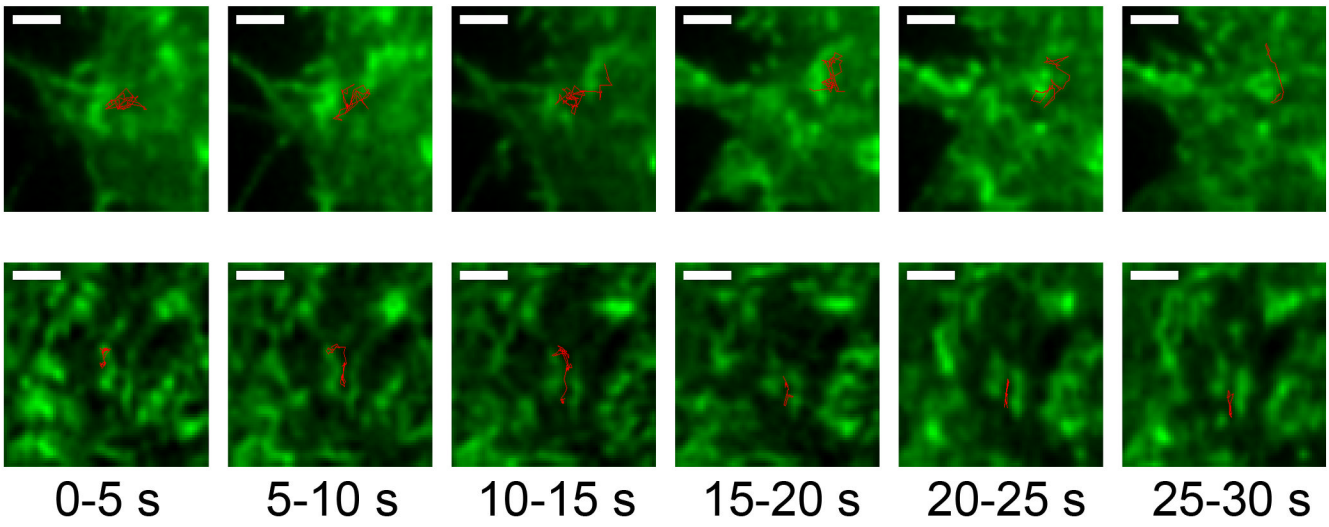


Figure 1. SPT reveals dynamic actin corrals

Trajectories (red) of single QD655-IgE bound FcεRI can be tracked with respect to the underlying actin (green) in RBL cells expressing GFP-actin. The actin structures are dynamic on the timescale of ~1–10 seconds. Shown are two trajectories (red) broken into 5 second intervals. The actin image is the mean value of the 5 second interval. Scale bar = 1 μm. Unpublished images courtesy of Keith Lidke and similar to those found in Andrews *et al*[5].

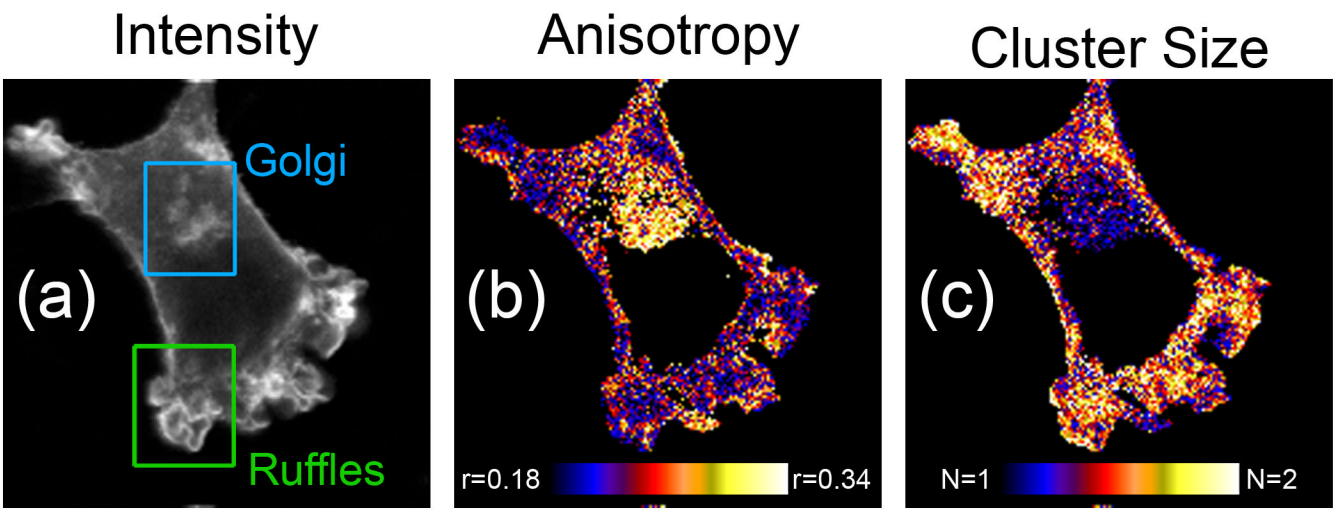


Figure 2. Homo-FRET imaging provides a map of protein aggregation

(a) Intensity, (b) anisotropy (r) and (c) cluster size (N) images of NIH3T3 cell expressing GPI-GFP. Homo-FRET imaging reveals that GPI-GFP is found as small clusters in ruffles on the plasma membrane, but remains monomeric in the Golgi. Images courtesy of Arjen Bader and Hans Gerritsen and reproduced with permission from Bader *et al*[23].

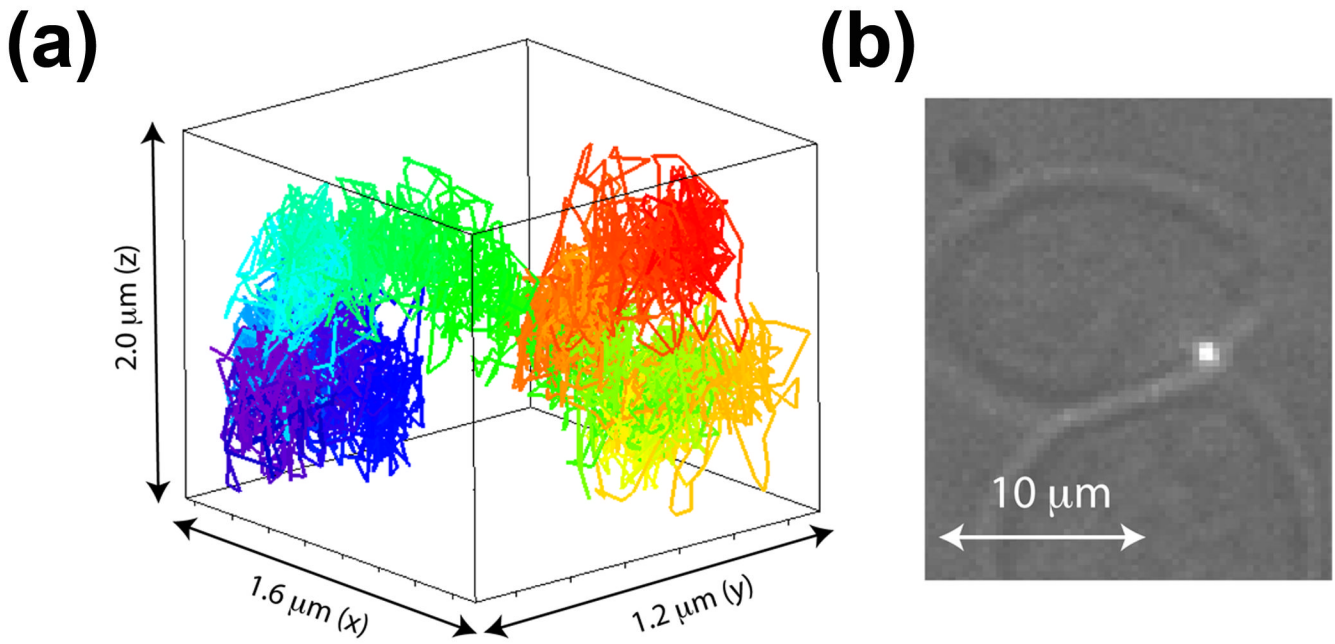
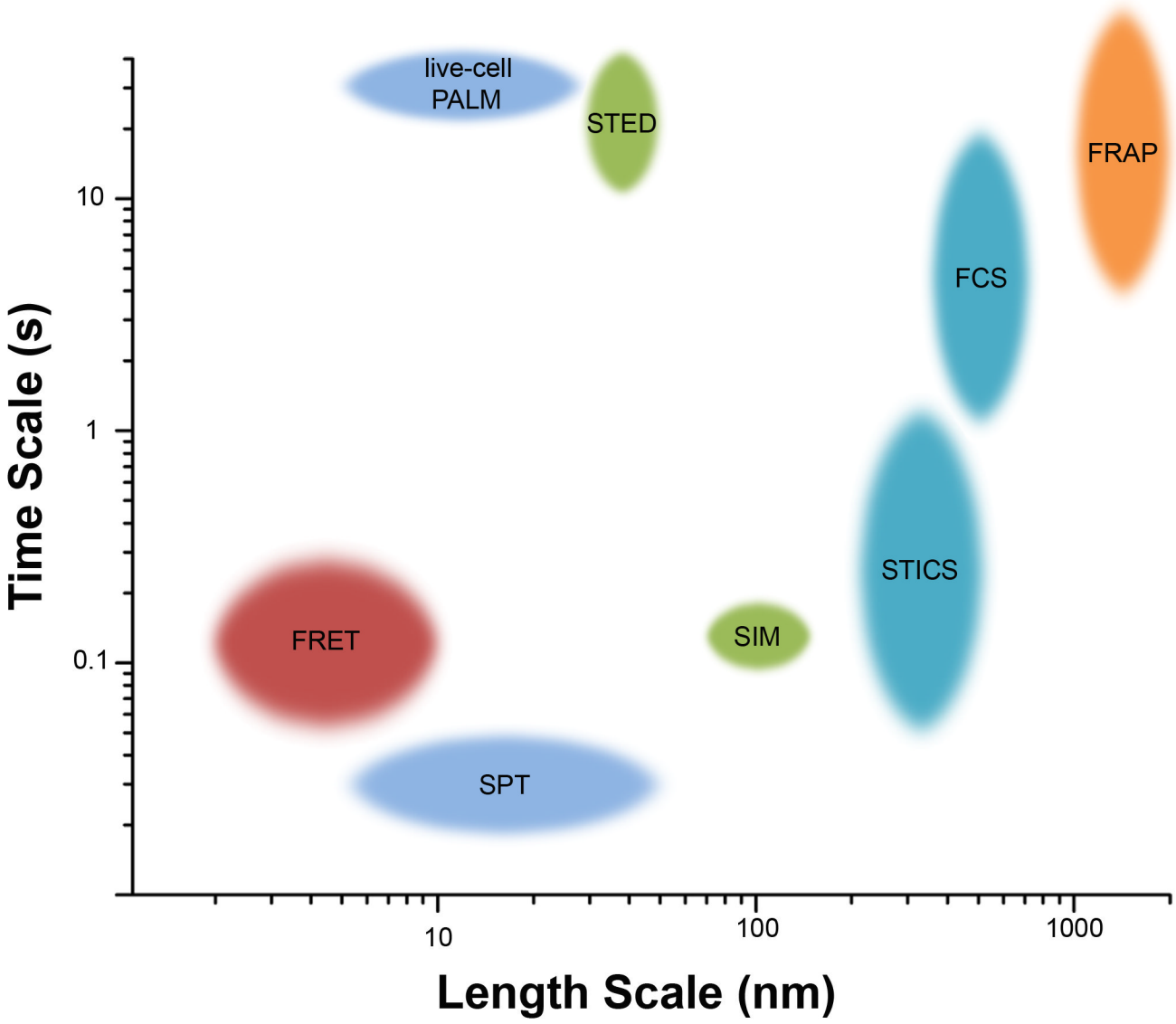


Figure 3. 3D tracking of membrane protein motion

(a) 3D trajectory of QD-IgE-FcεRI diffusion along the side of the plasma membrane for 40 s. Trajectories are color coded as a function of time from start (red) to finish (violet) following the rainbow (ROYGBIV) scheme. (b) Transmission image of cell overlayed with the QD emission (white spot) to show the location of the tracked receptor. Unpublished figure courtesy of James Werner and similar to those found in Wells *et al*[47].



Box 1 Figure 1. Comparison of length and time scales accessible by fluorescence microscopy techniques

Length scales refer to resolution (STED, SIM), localization accuracy (PALM, SPT), distance over which interactions can be detected (FRET), or the limiting size of the measurement field (FCS, ICS, FRAP). The time scale refers to the amount of time to complete one measurement, representing the maximum rate at which dynamic *changes* in the sample can be detected. In most cases, slower events and longer length scales can also be detected. Size of oval approximates the typical range of length and time scales in live cell imaging. Similar techniques are grouped by color.

Box 1 Table 1

Comparison of fluorescence microscopy techniques

Method	Measurables	Advantages	Limitations
FRAP [8,9]	• Average protein diffusion • Mobile/immobile fraction	• Standard on any confocal microscope	• Measures average behavior of entire population • Low spatial and temporal resolution • Requires bleaching with high intensity laser light
FRET [1,18,19]	• Distance between donor and acceptor labels	• Typical range is 1–10 nm • Reports interactions between two labeled proteins or conformational changes within a dual-labeled protein	• Requires tagging of proteins with appropriate fluorophores • Potential for false-negative results
Homo-FRET [21,63]	• Protein aggregation state - based on anisotropy measurements	• Single class of fluorphore needed	• Anisotropy measurements require special equipment
SPT [5,8,9,24,25]	• Trajectories and diffusion of individual proteins • Reveals different modes of motion (free, restricted, immobile)	• nm spatial and ms time resolution • Multi-color SPT allows for distinguishing between multiple protein species	• Slower proteins are easier to track • Generation of monovalent probes is non-trivial • Low labeling density required
FCS/FCCS [28–30,64,65]	• Protein diffusion coefficients • Protein-protein interactions • Protein concentration	• Live cell studies	• Requires special detectors • Measurements are slow (>10s) and made at a fixed point in the cell • Not applicable for immobile proteins • Limited concentration range
ICS/ICCS [31]	• Protein number density and aggregation state	• Acquired with standard laser scanning confocal microscope or TIRF set-up • Can be applied to samples of low or high concentration	• Measures average protein behavior, subpopulations are not distinguished
RICS/ccRICS [31,36,66]	• Provides a spatial map of protein mobility and protein interactions	• Acquired with standard laser scanning confocal microscope • Can measure fast dynamics (μs-ms) • Live cell imaging	• Scan rate must be comparable to the diffusion being measured • Assumes diffusion of proteins in a homogeneous medium • Immobile species may mask diffusing particles, proper filtering of the images can correct this
N&B/ccN&B [35,67]	• Brightness and number of molecules in each pixel	• Live cell imaging • Possible to determine immobile proteins • Can be measured with standard laser scanning confocal microscope	• Assumes that the intensity fluctuations are due only to fluorescent molecules • Photobleaching needs to be accounted for
STED [52,55,56,68]	• Super-resolution images (~ 40nm)	• Live cell imaging possible	• Requires expensive, specialized equipment
Localization Microscopy [48,52,53,59–61]	• Super-resolution images (down to 10 nm)	• Live PALM has been achieved with 60 nm and 25 s resolution	• Ultimate spatial resolution requires fixed samples • Data acquisition time can be long (min to hrs)