CHAPTER

4

High Spatiotemporal Bioimaging Techniques to Study the Plasma Membrane Nanoscale Organization

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INTRODUCTION

A large variety of signal transduction processes are initiated by proteins or lipids located at the plasma membrane. Binding affinity of receptors for their ligands and changes in receptor expression levels are not enough to explain the fine-tuning and integration of cell signaling responses. It is by now well established that regulation of signaling threshold and duration also requires local nanoscale changes in receptor lateral organization and mobility within the plasma membrane.[1,2]

Studies performed in the early 1990s by Jacobson, Kusumi, Edidin, and others pioneered techniques such as fluorescence recovery after photobleaching (FRAP), single-particle tracking (SPT), and optical laser trapping to study for the first time the lateral diffusion characteristics of several membrane components.[3–5] Their observations clearly demonstrated that membrane proteins do not exhibit the continuous, unrestricted random diffusion that was expected from the fluid mosaic model proposed by Singer and Nicolson back in 1972.[6] Instead, they revealed that many proteins diffused in various and complex ways that clearly pointed toward the existence of lateral heterogeneity in the membrane structure, thus challenging the Singer and Nicolson model.[7] New models were proposed that would explain this heterogeneity, such as the membrane-skeleton fence model[3] or the transient confinement zone concept.[8]

A few years later, in 1997, the concept of lipid rafts was put forward that literally revolutionized the cell membrane field by proposing the existence of microscale lipid platforms (rafts) within the plasma membrane that would transport selected proteins or function as relay stations for intracellular signaling.[9] Almost at the same time, a seminal publication by Rubinstein and colleagues[10] demonstrated the existence of another type of membrane organization mediated by the lateral interactions between various membrane receptors and members of the tetraspanin family, which gave rise to a network of molecules also known as the tetraspanin web. Over the next several years, both tetraspanins and lipid rafts were the subject of some controversy, mainly fueled by the elusive nature of lipid rafts[11,12] and the apparent redundancy among tetraspanin molecules.[13,14] However, both concepts have ignited development of a completely new area in cell biology and have revitalized interest in the plasma membrane, forcing the scientific community to reevaluate the fluid mosaic model developed almost three decades earlier by Singer and Nicolson.[6]

The following years witnessed a rapidly growing number of publications trying to reconcile all of these concepts, and it is by now well established that nanoscale compartmentalization occurs at the plasma membrane but its underlying mechanisms are more complex and diverse than a simple division in rafts, non-rafts, or tetraspanin web (Figure 4.1). Lipid–lipid, protein–protein and lipid–protein interactions have all been demonstrated to contribute to the formation of spatiotemporally dynamic compartments within the cell membrane.[15] An elegant example of how lipids can regulate membrane receptor dynamic and function is provided by the study by Coskun and colleagues,[16] which revealed that the compositional diversity of the surrounding lipid membrane specifically modulates the signaling capacity of the human epidermal growth factor receptor (EGFR). Our laboratory recently

Fluorescence Microscopy
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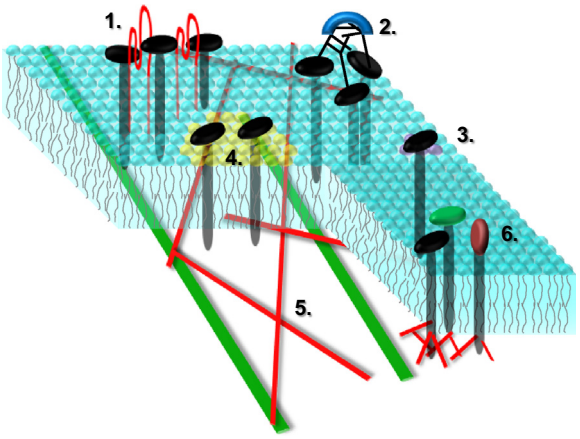

FIGURE 4.1 **Nanoscale organization of the plasma membrane.** The nanoscale organization of proteins within the plasma membrane can be regulated by (1) protein clustering via protein–protein interactions (e.g., tetraspanins[14]); (2) protein clustering via glycosylation; (3) protein–lipid interactions; (4) confinement in lipid-raft like domains; (5) restricting or promoting lateral segregation of membrane molecules via the cytoskeleton, either actin corrals or microtubules; and (6) dynamic patterning by nanometric-sized subcortical actin asters.[22]

demonstrated the involvement of protein–protein interactions in modulating the formation of efficient virus-binding nanodomains by the HIV-1 receptor DC-SIGN on antigen-presenting cells,[17] while Low-Nam and colleagues[18] showed how ligand binding can promote EGFR domain formation and stabilize receptor dimerization.

Another important player in shaping the heterogeneity of the plasma membrane is the cortical cytoskeleton. Cortical actin corrals as well as microtubules have been shown to play an important role in restricting or promoting lateral segregation of membrane molecules.[19,20] Besides the role of actin corrals in restricting protein diffusion, a novel theoretical framework has been recently proposed by the laboratories of Rao and Mayor[21,22] in which nanometric-sized subcortical actin asters dynamically pattern the membrane organization. They proposed that membrane nanoclustering is based on active hydrodynamics, wherein molecules bound to short, dynamic, polymerizing actin filaments at the cortex are actively driven to form transient clusters. Although the existence of these nanometric actin filaments is still only a key assumption of this theoretical framework and has not yet been directly proven by imaging techniques, this new model provides an active mechanism for the spatiotemporal regulation of the molecular organization at the cell membrane.[21,22]

Over the years it became more and more clear that the existing biochemical tools were not sufficient to unravel the principles of the membrane nanoscale organization. To fully determine protein–protein interactions and comprehend the regulatory role of lipids and membrane, it is imperative to study signaling events in living cells at high temporal and spatial resolution. The exploitation of advanced fluorescence-based bioimaging techniques to study membrane organization and the constant optimization of image analysis algorithms have indeed been instrumental in supporting biochemical claims and have significantly advanced our understanding of the plasma membrane landscape at the nanoscale.[23]

Advancements in fluorescence microscopy techniques have made it possible to evaluate the protein aggregation state and the dynamics and interactions of living cells and to measure biochemical parameters *in situ*. Image correlation techniques can determine aggregation state and the average mobility of proteins as well as protein–protein interactions based on ensemble measurements. Imaging techniques that can circumvent the diffraction limit, including near-field scanning optical microscopy (NSOM), stimulated emission depletion (STED), and localization microscopy, are capable of directly revealing the nanoscale organization of the membrane.[24,25] Single molecule imaging, such as Förster resonance energy transfer (FRET) imaging and multicolor single particle tracking (SPT), provides a view of protein dynamic behavior at the molecular level, making it possible to discriminate between fractions of molecules with different rates of mobility.[26] In this chapter, some of these key microscopy approaches are addressed, with an emphasis on both advantages and disadvantages, and the chapter describes some more recent developments that hold great promises for the field of membrane biology.

SPATIAL RESOLUTION

The term *resolution* indicates the ability to distinguish two separate items from one another. Resolution can be quantified as the smallest distance between two objects in which the objects still appear to be separate. The resolution that can be achieved by light microscopy is directly related to the wavelength of the light. This phenomenon is known as the *diffraction limit* and originates from the fact that it is impossible to focus light on a spot smaller than roughly half its wavelength.[27] Because the wavelength of blue light is 400 nm, the theoretical resolution of a conventional optical microscope can never be higher than 200 nm, meaning that it is impossible to resolve two fluorescent objects that are separated by less than 200 to 250 nm. The discovery of a compartmentalized organization of membrane proteins and lipids clearly required imaging techniques that would circumvent the diffraction limit issue, allowing exploration of the plasma membrane at the nanoscale (Figure 4.2).

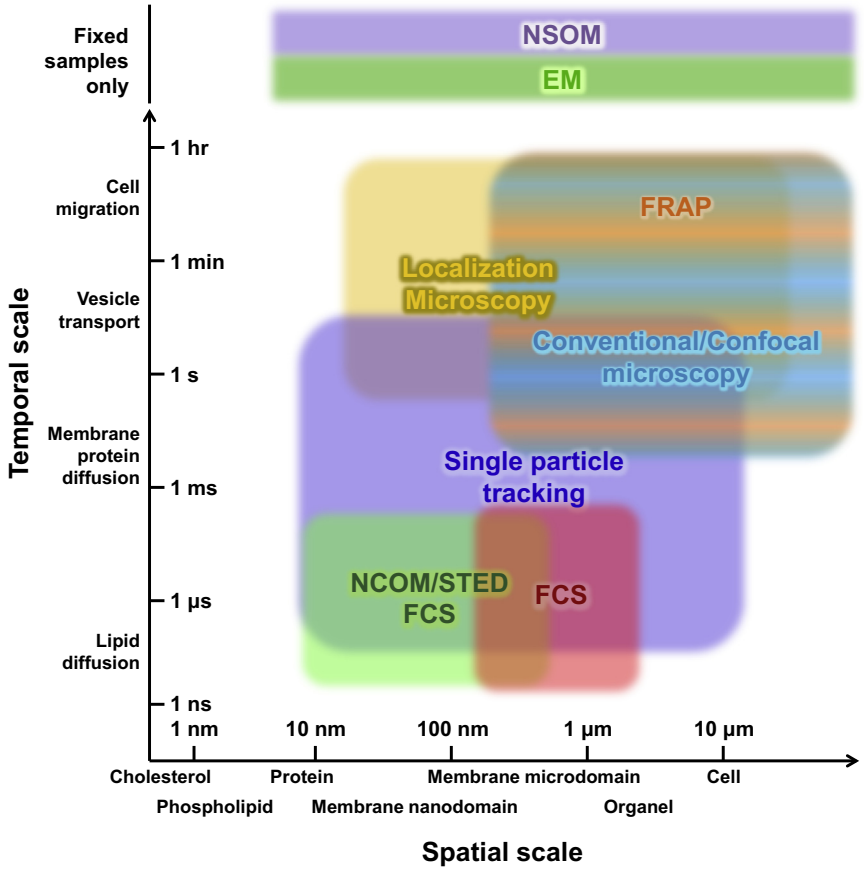

FIGURE 4.2 **Spatiotemporal resolution of bioimaging techniques.** The spatial and temporal scales that can be resolved with bioimaging techniques are shown. Each technique is represented by a colored square. The axes show the spatial and temporal scales and corresponding biological structures and processes. Abbreviations: EM, electron microscopy; FCS, fluorescence correlation spectroscopy; FRAP, fluorescence recovery after photobleaching; NSOM, near-field scanning optical microscopy; STED, stimulated emission depletion.

Transmission Electron Microscopy

Transmission electron microscopy (TEM)[28] combined with whole-mount gold labeling of membrane receptors on fixed intact cells provided an excellent solution. TEM mirrors its optical counterparts except that it uses a focused beam of electrons instead of light to examine specimens on a very fine scale. The image of the specimen is the result of beam electrons that are differentially scattered by the various features of the specimen versus those that are not and are eventually transmitted onto a screen. In order to visualize specific proteins in a biological sample, immunogold EM is performed. This consists of labeling the desired protein with specific primary antibodies, followed by conjugating secondary antibodies to colloidal gold particles. Gold is highly electron dense and will appear as black dots on the image. Immunogold EM can be classically performed on thin sections but it can also be applied to the visualization of features in intact cells, the latter approach being known as whole-mount. This method has been successfully applied by several groups in the past, including ours, to determine the spatial distribution of several membrane proteins such as the potassium channel Kv1.3,[29] the IL-2 receptor,[30] the leukocyte-specific integrin LFA-1,[31] and the HIV-1 receptor DC-SIGN.[32] In fact, not only have transmembrane proteins been labeled and imaged by whole-mount TEM but also the nanoscale organization of proteins anchored to the inner leaflet of the plasma membrane has been investigated using the so-called native membrane sheets.[33] The approach involved sandwiching of cells between glass coverslips and EM grids, followed by ripping, fast fixation, and specific labeling with functionalized nanoprobes. In this way, the gold-labeled proteins can be analyzed with respect to membrane features such as clathrin-coated pits, caveolae, and the cortical cytoskeleton.[34–39] In all cases, from the digitized EM images the x,y coordinates of the gold particles can be extracted. This combined with spatial point pattern analysis tools allowed reconstruction of the spatial distribution of both outer and inner membrane proteins.[40] In this way, high-resolution topographical maps of colloidal gold nanoparticles were generated that labeled receptors and

signaling proteins in cellular membranes. However, the limited possibility of performing double or triple staining using gold particles, the possible artifacts introduced by the harsh fixation and dehydration steps, and the lack of dynamic information represent the major limitations of the whole-mount TEM approach.

Near-Field Scanning Optical Microscopy

Some of the TEM drawbacks can be overcome using NSOM (Figure 4.3), a scanning probe microscopy technique that combines the favorable features of fluorescence microscopy with superior spatial resolution and topographical information, being particularly well suited to study both model and biological membranes.[24,41] In single-molecule NSOM, an optical fiber with a nanoscale aperture is raster scanned onto a sample kept in close proximity (within 10 nm) to the probe. As a result, both topographic and optical images are simultaneously obtained, revealing the spatial distribution of fluorescent molecules. In the past few years, remarkable progress has been made in the application of NSOM to image the distribution of cell membrane molecules, increasing our understanding of how cells organize surface receptors into nanodomains modulating their functional state. Pioneering studies on the application of NSOM to investigate membrane receptors visualized nanometric-sized domains of the HIV-1 receptor DC-SIGN on the membrane of antigen-presenting cells both in air and in liquid.[42] Other publications appeared almost at the same time reporting NSOM imaging of immunostained receptors at the plasma membrane of cardiac myocytes.[43,44] These studies revealed that the voltage-gated L-type Ca^{2+} ion channel is organized in small clusters with an average diameter of 100 nm,[44] while the beta-adrenergic receptors are organized in multiprotein complexes of about 140-nm average diameter, of which 15 to 20% is preassociated with caveolae and sufficient to exert changes in the ligand-induced myocyte contraction rate.[43] Dual-color NSOM was subsequently exploited to determine the degree of nanoscale colocalization between the receptors for interleukin-2 and -15 at the cell surface of human CD4+ T cells,[45] as well as the organization and mutual localization of the asialo GM1 sphingolipid with caveolae.[46] More recently, by using NSOM in collaboration with Garcia-Parajo's laboratory, our group demonstrated that only 25 to 30% of the nanodomains of the putative raft-associated integrin LFA-1 reside within 150 nm from a small fraction of oligomeric GPI-anchored proteins, thus forming hotspots for the initiation of cell adhesion.[47] Furthermore, by NSOM imaging, we unraveled the existence of a raft-based compositional interconnectivity among GM1, GPI-anchored proteins, and LFA-1 at the nanoscale that is likely to mix minimal raft coalescence units into large-scale rafts upon specific activation stimuli.[48]

Despite the potential of NSOM to address this type of biological questions, the major limitation of this approach was its slow scanning speed, which prevented its use in live cell systems. Recently, however, a combination of NSOM and fluorescence correlation spectroscopy (FCS) allowed performing nanoscale fluorescence correlation spectroscopy on plasma membranes of living cells, revealing details of the fast diffusion of fluorescent lipid analogs that were very comparable to those obtained by STED-FCS,[49] thus paving the way to future applications of NSOM to study dynamics of membrane components in living cells.[50]

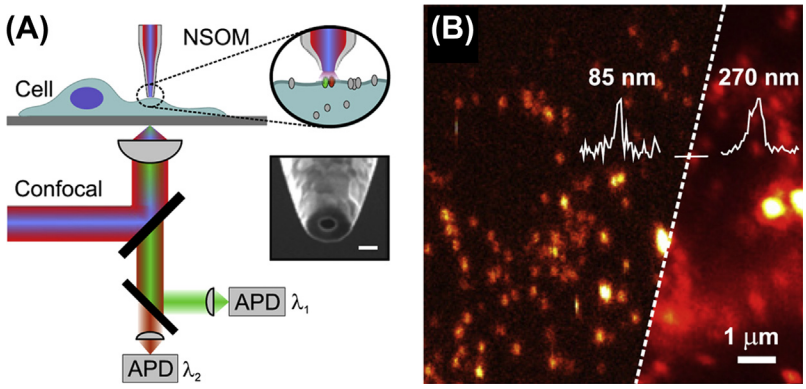

FIGURE 4.3 **Principles of near-field scanning optical microscopy.** Near-field scanning optical microscopy (NSOM) reveals membrane nanoscale organization. (A) Principle of operation of a NSOM setup built on a confocal microscope. In NSOM, an Al-coated hollowed optical fiber is a probe that raster scans laterally a sample while locally exciting fluorophores exclusively at the cell surface. The small inset shows a typical NSOM probe with an aperture smaller than 100 nm. Scale bar is 100 nm. (B) Composed image of confocal microscopy (right) and NSOM in liquid (left) of the integrin LFA-1 specifically labeled with a fluorescent antibody at the cell surface of fixed monocytes. The superior resolution of NSOM (85 nm) over that of the confocal microscope (270 nm) is emphasized by the line profile over the same individual fluorescent feature. *Images are modified from van Zanten et al.*[47]

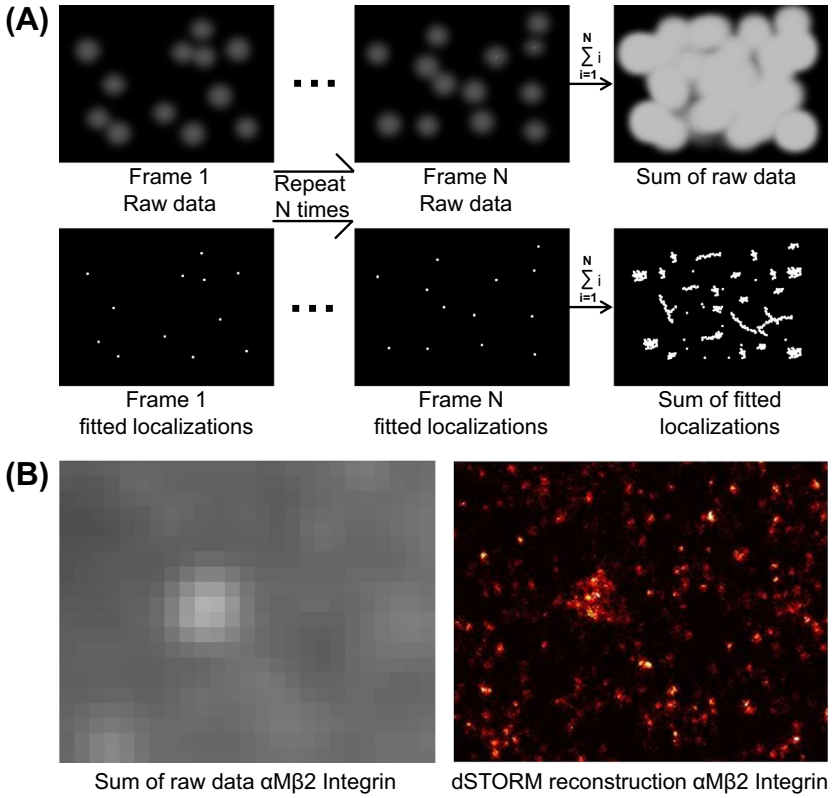

FIGURE 4.4 **Localization microscopy.** (A) The acquisition procedure of localization microscopy data. A sparse subset of fluorescent molecules is imaged (top) and their location is determined by fitting the center of their emission peak to a Gaussian profile (bottom). Next, these molecules are bleached or return to a dark state, and another subset of molecules is imaged. This process is repeated until a complete image is reconstructed from all of the individual localizations. (B) An example of a localization microscopy image and the corresponding diffraction-limited image of an αMβ2 integrin immunolabeling acquired by dSTORM.

Localization Microscopy

One way to image cells with nanometer precision is localization microscopy. This method has been simultaneously developed by three research groups and relies on localizing individual fluorophores with accuracy up to 20 times better than the diffraction limit.[51–53] The resolution of light microscopy is determined by the point spread function (PSF) of the microscope, which is the blurry spot one fluorophore gives in an image. An image of multiple fluorophores, like a cell labeled for a certain protein, is a convolution of the PSF with the locations of each fluorophore present in the sample, which does not allow resolving the locations of individual fluorophores. However, if only one fluorophore is excited and detected, this will show up as the PSF in the image and the center of this spot can be determined with a precision up to 10 nm, depending on the number of photons detected.[54] Therefore, by detecting each fluorophore sequentially, their locations can be determined very accurately. All of the methods of localization microscopy rely on this principle. To generate a super-resolution image, a sparse subset of fluorescent molecules is imaged at any given time, and their location is determined by fitting the centroid of their emission intensity peak to a Gaussian profile. Next, these molecules are bleached, and another subset of molecules is imaged. This process is repeated until a complete image is reconstructed from all of the individual localizations (Figure 4.4). To image only a subset of molecules, the fluorophore must be able to occupy two different states, of which only one is detected under the imaging conditions. The different localization microscopy methods use different probes and strategies to achieve and control these two states.[55] Both photoactivated localization microscopy (PALM) and stochastic optical resolution microscopy (STORM) use photoswitching of fluorescent probes between a visible (ON) state and an invisible (OFF) state. PALM uses genetically encoded fluorescent proteins that can be photoactivated or photoswitched,[51,52] whereas STORM uses tandem organic fluorophores that can be photoswitched.[53] Direct STORM (dSTORM) exploits the spontaneous switching of individual organic fluorophores between a visible and a dark state.[56] Finally, in ground state depletion microscopy followed by individual molecule return (GSDIM), organic dyes are forced into a dark triplet state, and their stochastic return to the visible ground state is utilized to reconstruct the image.[57]

To localize all molecules in a field of view, many images containing only a few detected fluorophores must be sequentially acquired. This can take up to a few minutes, making localization microscopy an inherently slow method that was initially limited to fixed cells. However, advances in camera technologies have allowed higher imaging rates, which combined with new analysis

methods have led to the recent application of localization microscopy to live cell imaging.[58–60] Initially, localization microscopy was limited to two-dimensional localization of fluorescent probes. Recently, several methods have been described to also determine the *z*-position of each fluorophore, thereby obtaining three-dimensional information about molecular structures. Various approaches to obtain this three-dimensional information have been used, including the use of a PSF that is asymmetrical in the *z*-dimension,[61,62] splitting different *z*-planes onto different regions of a detector,[63] or by the use of interferometry.[64,65] Among various biological systems, the three-dimensional structure of clathrin-dependent endocytic vesicles has been revealed by using such a three-dimensional localization approach.[61,66]

To assess the molecular organization of proteins in cell membranes with localization microscopy, a quantitative description and evaluation of the localized particles are essential. Multiple methods have been described that enable quantification of molecular organization, including quantification of clustering, cluster size, and density.[55] One method that quantifies clustering or co-clustering is Ripley's K-test,[67,68] which quantifies the amount of clustering as a function of length scale and information about the degree and spatial scale of molecular clustering. Moreover, Ripley's K-test can be used to generate a map of the degree of clustering over different regions of the plasma membrane.[69]

Another method to quantify the spatial organization of membrane proteins is pair-correlation PALM (PC-PALM).[70] This method takes into account that each fluorophore can be detected multiple times, as a result of blinking or detection of the same fluorophore in consecutive frames; it uses pair correlation algorithms to calculate the contribution of multiple localizations of the same fluorophore. This allows an accurate assessment of different physical parameters of protein distribution. PC-PALM has been used to study the clustering of various membrane proteins under steady-state conditions and after perturbations of membrane lipid composition and the cortical actin cytoskeleton.[71] In particular, co-clustering of the transferrin receptor with clathrin-coated pits[72] and the colocalization of cross-linked GPI-anchored proteins with the cortical actin network[71] have been revealed.

Localization microscopy can easily be combined with total internal reflection fluorescence (TIRF) illumination, making it an ideal method to resolve the organization of the cell membrane. Using PALM on live and fixed cells, Hess and coworkers[59] assessed the spatial distribution of membrane protein hemagglutinin (HA) from influenza virus and showed that the protein exists in elongated clusters of ~40 nm, thereby confirming the earlier results obtained with electron microscopy.[73] More recently, using dual-color PALM, the HA clusters were shown to colocalize with actin-rich membrane regions, and an intact cortical actin network was proven essential for HA cluster organization.[74]

One of the best described examples of a highly organized membrane structure is represented by the immunological synapse (IS), the complex cell–cell contact area between an antigen-presenting cell and a T lymphocyte.[75,76] Many efforts have been made to unravel the nanoscale organization of the IS using localization microscopy. Initially, it was proposed that the IS has a bull's eye pattern with a central supramolecular activation cluster (cSMAC), where signaling is initiated and which is enriched for the T-cell receptor (TCR) complex, surrounded by a peripheral SMAC (pSMAC), where adhesion proteins are localized.[75,76] Later, the importance of microclusters composed of the TCR complex and co-stimulatory and signaling molecules and formed before establishment of a cSMAC and a pSMAC was recognized.[77] However, this vision of the IS organization has significantly changed due to the application of localization microscopy to study the IS at the nanoscale. Using a combination of immuno-EM and PALM, Lillemeier and coworkers[78] showed that, before the initiation of signaling, both the TCR and the adaptor protein Lat are present in nano-islands of 35 to 70 nm containing 7 to 30 TCRs, which upon TCR stimulation were shown to coalesce but not fuse. Using PALM and dSTORM, Williamson and coworkers[67] showed that translocation of subsynaptic Lat-containing vesicles to the T-cell surface resulted in an increase of Lat clusters after T-cell stimulation and that these newly recruited clusters of Lat, rather than preexisting Lat domains in the plasma membrane, were phosphorylated after TCR triggering. A combination of localization microscopy and lifetime imaging of membrane-order-sensitive dyes was used to show that Lat molecules associated with ordered lipid domains exhibit retarded mobility and decreased clustering.[79] Finally, Lck and Src, kinases involved in TCR signaling, were also found to be organized in subresolution-sized clusters that do not overlap.[68] Altogether, the application of localization microscopy to study the IS yielded unprecedented insight into the molecular organization of the lipid domains, receptors, and kinases involved in the initiation of T-cell signaling.

TEMPORAL RESOLUTION

Signaling events are initiated at the plasma membrane via interactions between individual molecules, and information about these interactions can be obtained by monitoring the lateral dynamic behavior of proteins within or proximal to the plasma membrane. Lateral transport and interactions of lipids and proteins occur on microsecond and millisecond time scales;

therefore, observing these interactions with a high temporal resolution is key to understanding how these processes are initiated and shape cellular responses to external cues (Figure 4.2). Below, we discuss some of the key microscopy methods exploited to study the plasma membrane organization at high temporal resolution.

Fluorescence Recovery after Photobleaching

Fluorescence recovery after photobleaching (FRAP) is a microscopy-based method used to study the mobility of fluorescent molecules that has been employed since the mid-1970s to assess mobility of molecules in the cell membrane.[80–83] Thanks to the development of GFP fusion proteins, a revival of FRAP applications was seen in the mid-1990s.[84] In FRAP, fluorescent molecules in a region of interest (ROI) within the plasma membrane are bleached by a high-intensity laser source. If the fluorescent species is mobile, the bleached molecules within the ROI will be exchanged by fluorescent molecules diffusing from surrounding regions. The resulting recovery of fluorescent intensity in the ROI is monitored and plotted on a recovery curve, which contains information about the fraction of molecules that are mobile and the half time of recovery (Figure 4.5). The curve can be fitted to an appropriate model for the behavior of the fluorescent species, and models that account for diffusion, interaction, and binding behavior are readily available.[85–88] FRAP is usually implemented in confocal microscopes, thus limiting its spatiotemporal resolution to that of the respective microscope. However, the availability of confocal microscopes combined with a relatively straightforward experimental approach have made FRAP the method of choice for many studies of membrane dynamics. FRAP has been used to investigate membrane heterogeneity, a concept that was inferred from the observation that FRAP curves from lipids and membrane proteins contain an apparent immobile fraction (Figure 4.5).[89–91] Also, FRAP has been used extensively to assess the raft hypothesis, but due to the lack of spatial resolution and the ensemble nature of the method, FRAP could not provide definitive information about the existence or nonexistence of raft microdomains.[92,93] Recent advances in this methodology include the development of models to separate lateral diffusion from membrane recruitment of non-integral membrane proteins. These methods rely on measuring the intensity in the bleached ROI in the temporal dimension and use the spatial intensity distribution within the bleached ROI to gain information about the type of mobility of molecules.[94–96]

Förster Resonance Energy Transfer

The response of a cell is the result of various rate-controlling steps at different levels in the signaling cascade, starting at the plasma membrane with ligand-receptor binding followed by interactions of the activated receptor and its intracellular effectors. The rate kinetics of protein interactions can be determined using Förster resonance energy transfer (FRET)-based techniques.[97,98] When two fluorophore-labeled proteins are

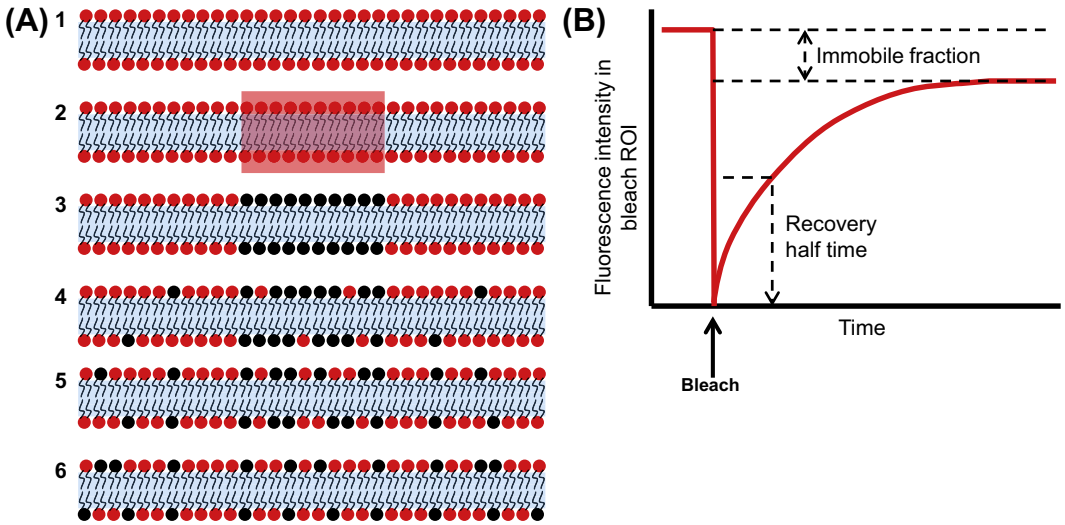

FIGURE 4.5 **Fluorescence recovery after photobleaching.** (A) A membrane containing a fluorescent species is imaged (1). The fluorophores in ROI within the field of view are photobleached by a high-intensity laser source (2, 3). When the fluorescent molecules are mobile, the bleached molecules will redistribute throughout the membrane and the intensity in the bleach ROI will recover by exchange of bleached and unbleached molecules (4–6). (B) Fluorescence recovery curve showing the intensity over time in the bleach ROI. From the recovery curve the immobile fraction and recovery half time can be determined.

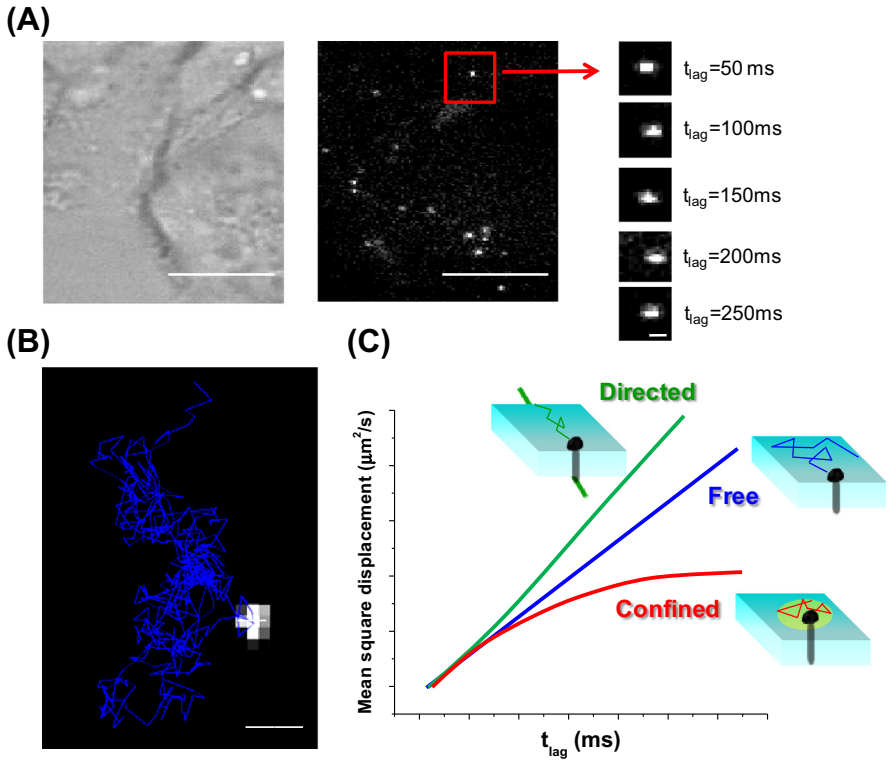

FIGURE 4.6 **Single-particle tracking to determine receptor dynamic behavior.** (A) Left image depicts the apical membrane of HEK293 cells transfected with the HA-tagged receptor EP2. Right image is the corresponding fluorescence image showing the signals from quantum dots (QDs) that were monovalently conjugated to anti-HA Fab fragments, which allowed labeling of the HA-tagged EP2 in living cells. To the right a zoom of multiple frames of one QD is depicted. (B) In each image/frame of the movie, automated analysis yielded values for the integrated fluorescence signal and the lateral position of the labeled receptors with high accuracy (~16 nm),[39] allowing accurate reconstruction of trajectories from the positional shifts of the labeled receptors in consecutive images. (C) Analysis of the receptor trajectories results in the mean square displacements (r_i2) versus time lag (t_{lag}) plots which can be fitted using models describing different modes of diffusion. The heterogenic microscopic organization of the plasma membrane imparts different types of motion onto receptors, such as transient confinement (red line) in dynamic microdomains, free Brownian diffusion (blue line), and directed flow (green line).

in close proximity (<10 nm), the donor fluorophore will transfer energy to the acceptor fluorophore. The efficiency of the energy transfer can be measured in a few ways (reviewed in detailed in Zeug et al.[99]): (1) by detecting changes in the intensity of the acceptor emission or the ratio of the intensity of the donor and acceptor emission; (2) measuring changes in the lifetime of the donor (FLIM), which will decrease in the presence of an acceptor; and (3) quantifying changes in the photobleaching rates of the donor, which shows a longer decay in the presence of the acceptor.[100] Utilizing the measured efficiency allows for the determination of direct interactions between proteins in live cells, in addition to interactions found by biochemical approaches, such as co-immunoprecipitation. For example, FRET analysis showed that MT1−MMP collagenolytic activity is regulated via lateral association with the tetraspanin CD151, thereby forming a α3β1 integrin/CD151/MT−MMP ternary complex in the plasma membrane.[101] The kinetics of receptor-mediated signal transduction were also measured using high-speed FRET analysis between the fluorescently labeled α(2A)-adrenergic receptors and the fluorescently labeled subunits of the G protein, leading to the hypothesis that α(2A)-adrenergic receptors interact with G-proteins by rapid collision coupling.[102] Furthermore, conformational changes of a signaling protein[98] or between subunits of a signaling protein[97] can be monitored as an indication of activation, and in live cells these techniques are often used to study the kinetics of these interactions or conformational changes.

Single Particle Tracking

Signaling events at the plasma membrane are initiated via interactions between individual molecules, and information about these interactions can be obtained by monitoring the lateral dynamic behavior of proteins within or proximal to the plasma membrane. Because these interactions can be highly dynamic and tightly regulated in space and time, bulk studies such as protein biochemistry or conventional confocal microscopy will only yield information on the average properties of the dynamic behaviors and are compromised by poor time resolution. Single particle tracking (SPT) in living cells is a key approach to directly monitor protein dynamics within its natural environment. SPT uses the same principle as localization microscopy, where the center of mass of the PSF is determined with a positional accuracy up to 10 nm, depending on the number of photons detected. However, in order to study protein mobility, it is mandatory to monitor individual spots (<1 μm^2),[103] which requires labeling at suboptimal conditions, and is completely contrary to localization microscopy (Figure 4.6A). In addition, studying protein mobility requires high temporal resolution; therefore,

wide-field microscopy is the method of choice, because it allows simultaneous imaging of the entire area of view. Consequently, the time resolution of this imaging technique is only limited by the acquisition speed of the camera system and the fluorescence properties of the labeled species. TIRF microscopy is advantageous over conventional wide-field microscopy, as it limits the excitation volume to a region close to the coverslip surface, thereby reducing out-of-focus noise (autofluorescence or fluorescence from internalized fluorophores) and improving the spatial resolution without compromising acquisition speed. However, TIRF is limited to imaging dynamics at the basal plasma membrane, where protein mobility might be hindered by mechanisms controlling cell attachment (cytoskeletal network, integrins, etc.). Therefore, the choice of wide-field microscopy versus TIRF to perform SPT depends on the biological questions one wants to address, and appropriate labeling techniques have to be chosen with stronger fluorophores (e.g., quantum dots) for conventional wide-field microscopy to overcome out-of-focus noise. Analysis of the molecule tracks obtained with SPT (Figure 4.6B) provides unprecedented detailed information about mobility, membrane-binding lifetimes, and domain formation, all important determinants of signaling function (Figure 4.6C).[104–106] For example, the heterogeneous microscopic organization of the plasma membrane imparts different types of motion onto proteins, such as immobility, free Brownian diffusion, transient confinement in dynamic domains, hop diffusion across membrane picket fences, and directed flow.[107] Therefore, analysis of the trajectories and subsequent quantification of the mode of diffusion and the diffusion coefficient of molecules provide insight into the influence of the membrane nanoenvironment on signaling function.[108]

The power of SPT for signal transduction research has been convincingly demonstrated since the beginning of the century, yielding new insights into the dynamical processes occurring in receptor signaling. Starting in live cells, it was shown that amplification in signal transduction occurs, for example, through the epidermal growth factor (EGF)-induced clustering of the EGF receptor[109] or via increased mobility of the chemotaxis receptor[104] in combination with faster cycling of the ligand[110] at the leading edge of migrating cells. SPT of the FcRI receptor demonstrated that steps in receptor signaling occur via different levels of aggregation states.[31,111] These studies have also elucidated the mechanisms that can control the mobility, such as compartmentalization caused by cytoskeletal contacts via the actin cytoskeleton[19,112] or microtubules.[113,114] In addition, the lipid environment[115] and protein–protein interactions[116,117] have been shown to regulate receptor mobility. These mechanisms enable a cell to fine-tune its response through local changes in the rate, duration, and extent of signaling.

Although SPT analysis of the behavior of one molecule within the plasma membrane elucidates signaling events occurring in one protein, the direct visualization of signaling interaction events requires dual-color tracking of two proteins. However, due to the requirement of suboptimal labeling this is very challenging. Nevertheless, studies have utilized the tracking of two different colors to determine, for example, receptor dimerization.[105,118] In addition, various methods can be used to overcome the suboptimal labeling problem, such as single-molecule FRET, where one can track the donor and/or acceptor[119] and measure the mobility of a protein when it is interacting with another protein. Currently, multicolor single particle tracking is developed on a hyperspectral microscope, where up to eight colors of quantum dots can be tracked in hyperspectral images.[120]

Correlation Spectroscopy

Among the methods available to study the dynamics of cell membrane components, fluorescence correlation spectroscopy (FCS) has been used extensively to monitor the mobility of lipids and proteins in living cells. In general, in FCS, the sample is illuminated by a laser beam focused to a very small observation volume. For membrane measurements, the focus of a laser spot is a two-dimensional Gaussian detection area. The intensity of fluorescent molecules within this volume is monitored over time (Figure 4.7A). Both the diffusion of fluorescent species in and out of the observation volume and photochemical processes of the fluorescent dye will give rise to intensity fluctuations in time (Figure 4.7B). Because the photochemical processes, such as blinking and bleaching, occur at much shorter time scales than the diffusion, these processes can be separated. The dynamic properties of the fluorescent species can be analyzed using the autocorrelation function (ACF), which describes the self-similarity of the signal as a function of time lag τ. The ACF bears information about both the number of molecules and the average time the species resides within the observation volume (Figure 4.7C); by fitting the ACF with an appropriate diffusion model, these quantitative parameters can be extracted. Advantages of FCS include sensitivity for low concentrations, non-invasiveness due to low excitation energies, and accurate measurements of lipid and protein dynamics thanks to robust analytical tools. Moreover, one FCS measurement can be performed in a few seconds, allowing measurements of fast mobility changes. FCS theory, analysis methods, and experimental setups are reviewed extensively elsewhere.[121] Shortly after the initial description of the FCS, this

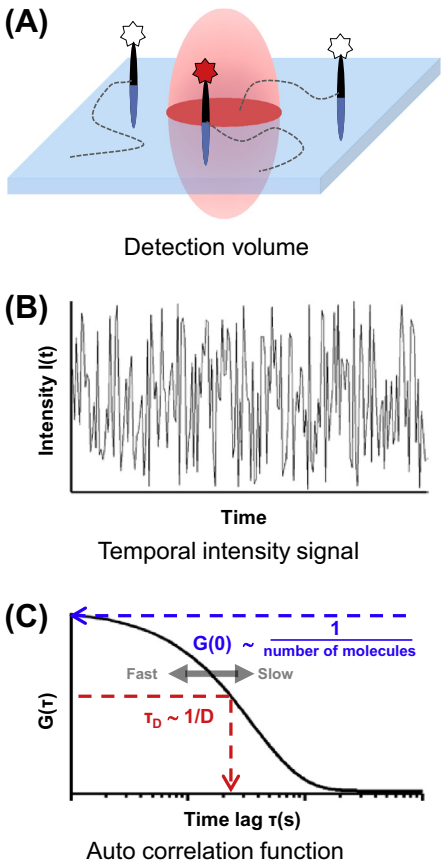

FIGURE 4.7 **Fluorescence correlation spectroscopy.** (A) A laser beam is focused on a membrane containing a fluorescent species. (B) Movement of fluorescent molecules in and out of the observation volume will give rise to temporal fluctuations in the fluorescence intensity. (C) The autocorrelation curve, calculated from the temporal fluorescence intensity signal, contains information about the concentration and diffusion of the fluorescent species.

method began to be applied to biological samples for the study of lateral diffusion of membrane proteins.[83,122]

An extension of FCS is fluorescence cross-correlation spectroscopy (FCCS), in which fluctuations of one fluorescent species are correlated with fluctuations of a spectrally distinct species. In this way, the interactions or correlations of dynamics between proteins or lipids can be assessed.[123,124] It is important to note that correlated dynamics of molecules in membranes do not require physical interactions but can also originate from co-confinement or coupling to cytoskeletal elements. FCCS has been used to study initiation of mast cell signaling at the membrane by cross-linking of FcεRI. Larson and colleagues[125] showed stimulation-dependent associations between fluorescently labeled IgE–FcεRI and Lyn–EGFP. FCCS was also used by Lillemeier and coworkers,[78] next to the localization microscopy described above, to show the correlation dynamics of the CD3ζ chain of the TCR complex and Lat.

In conventional FCS, fluctuations are measured in one illumination volume at a time. In scanning FCS (sFCS), a laser is scanned along a straight line or a ring, and the ACF is calculated in each spot along the line.[126,127] In this way, spatial variations in mobility can also be assessed. The time between subsequent observations for each volume is longer than that in conventional FCS, thereby reducing bleaching and making this method extremely suitable to study the dynamics of relatively slow species, such as membrane proteins.[128] Moreover, sFCS can be used to determine diffusion coefficients with high accuracy without the need to know the exact size of the measurement volume or calibration with a standard.[129] Also, it can be used in combination with two-color excitation to study molecular interactions.[130] So far, sFCS has primarily been applied to study lipid diffusion in model membranes,[126] but the feasibility of using sFCS for the study of live cells has been demonstrated.[128,129]

Due to cytoskeletal interactions, lipid membrane domains, or protein–protein interactions, the diffusional behavior of lipids and proteins in cell membranes is anomalous and usually best described by multiple species corresponding to the different populations undergoing different interactions. Both the spatial and the temporal scales of these behaviors can be assessed by spot variation FCS (svFCS),[131] in which the size of the illumination volume is varied and the transition time of molecules within the illumination volume is measured as a function of the volume size. Because the FCS volume in a membrane is a circular area, this results in a time versus area plot termed the *FCS diffusion law*. When molecules diffuse freely, this FCS diffusion law will be linear and upon extrapolation will cross the origin of the plot. However, if the molecules encounter barriers to diffusion or are trapped in a domain, the FCS diffusion law will no longer fit this model.[132] More specifically, the offset of the *y*-axis crossing of the curve can be used to accurately measure the trapping time of molecules in membrane nanodomains.[133] Using svFCS, Lenne and coworkers[134] showed that sphingolipid analogs, GPI-anchored proteins, and transmembrane proteins undergo transient confinements in cholesterol- and sphingomyelin-dependent microdomains. This study was extended by showing that these microdomains exist in various cell lines and primary cells. Moreover, they showed that sphingomyelin and cholesterol-dependent membrane microdomains were present in both the outer and inner leaflet, and that membrane recruitment and phosphorylation of the important kinase Akt were dependent on these domains.[135]

Fluorescence correlation spectroscopy can be combined with super-resolution microscopy approaches to decrease the focal spot size below the diffraction limit.[136]

Methods that have been used to perform subdiffraction FCS measurements are STED[137] and near-field excitation with NSOM probes.[50] Subdiffraction FCS has provided key insights into the dynamics of membrane lipids. Conventional FCS cannot directly resolve the anomalous diffusion of lipid species because the lipid nanodomains that impede lipid diffusion are much smaller than a diffraction-limited focal volume, and the diffusion of lipid species is very rapid compared to membrane proteins. Subdiffraction FCS was used to show that the phospholipid phosphatidylethanolamine (PE) exhibits homogeneous diffusion, whereas the sphingolipid sphingomyelin showed a broad distribution of residence times consistent with a cholesterol-induced confinement of sphingolipids.[50,137] This trapping in lipid nanodomains was further characterized using STED-FCS combined with the spot variation method.[137] Actin- and cholesterol-dependent trapping of lipids in subdiffraction lipid nanodomains was also demonstrated.[49]

Another way of analyzing FCS measurements is by looking at the distribution of the magnitudes of intensity fluctuations, a method known as fluorescence intensity distribution analysis (FIDA)[138] or photon counting histogram (PCH).[139] The method relies on determining the number of detected photons or "brightness" of the fluorescent species which provides information about size and stoichiometry of protein complexes. A modified form of FIDA has been used to detect clustering of eGFP-labeled EGF receptors in cells.[140] Using an extension of PCH known as number and brightness analysis, which is based on the analysis of sequential confocal microscopy images,[141] Nagy and coworkers[142] showed ligand-dependent and -independent dimerization and cluster formation of ErbB receptor subunits. These studies show the potential of FCS-based methods to assess receptor clustering in intact cells and elucidate the role of receptor oligomerization.

Image correlation spectroscopy (ICS) was developed as the imaging analog of FCS, in which spatial autocorrelation functions are calculated from images of fluorophores.[143] Unlike conventional FCS, ICS does not require fast diffusion of fluorophores, making it a suitable method to study membrane receptors and receptor clusters.[144,145] Several extensions of the FCS methods also have their ICS counterparts, such as image cross-correlation spectroscopy (ICCS) to study co-clustering of receptors and brightness or moment analysis to determine protein oligomerization states.[146,147] ICS-based methods have been applied to study membrane organization such as the partitioning of HA proteins in clathrin coated pits[148–151] and the mobility and confinement of H-Ras mutants.[152] Another method that is based on intensity distributions but not spatial correlations of fluorescence microscopy images is spatial intensity distribution analysis (SpIDA).[153] SpIDA is the imaging extension of the temporal PCH method and holds promise for the study of receptor oligomerization and trafficking.[154,155]

CONCLUSIONS AND OUTLOOK

High spatiotemporal resolution imaging has provided the cell biology field with valuable insights into the regulation of signaling by the nanoscale plasma membrane organization. However, investigating the local nanoscale changes in receptor lateral organization and mobility within the plasma membrane has required a trade-off between imaging the subdiffraction molecular organization or imaging the dynamics of coordinated interactions between proteins. Exciting advances are being made by accelerating acquisition in localization microscopy and merging with SPT to study the dynamics of individual proteins in the context of their environment, such as, for example, sptPALM.[60,156] High-speed localization microscopy and multiple particle tracking using hyperspectral microscopy allows the tracking of numerous subsets of proteins in succession and therefore observing heterogeneity in protein dynamics at different localizations in the membrane. Other advances are made by combining, for example, single molecule imaging with single molecule force spectroscopy (e.g., NSOM-FCS), allowing for the simultaneous measurement of localization, elasticity, and interaction of cell surface molecules. Furthermore, imaging methodologies being developed for fast three-dimensional tracking and three-dimensional super-resolution imaging are necessary for studying cellular events localized and proximal to the plasma membrane and subcellular structures such as cortical actin. Of course, as the optical tricks develop, the absolute need for novel or improved labeling techniques, fast and sensitive detection methods, and analysis algorithms, as well as the storage and handling of huge data files, is becoming more and more evident. We anticipate that in the coming decade the ongoing developments will enable true nanoimaging of signaling events in live cell membranes.

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