Review Article

Correlative nanophotonic approaches to enlighten the nanoscale dynamics of living cell membranes

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Dynamic compartmentalization is a prevailing principle regulating the spatiotemporal organization of the living cell membrane from the nano- up to the mesoscale. This non-arbitrary organization is intricately linked to cell function. On living cell membranes, dynamic domains or ‘membrane rafts’ enriched with cholesterol, sphingolipids and other certain proteins exist at the nanoscale serving as signaling and sorting platforms. Moreover, it has been postulated that other local organizers of the cell membrane such as intrinsic protein interactions, the extracellular matrix and/or the actin cytoskeleton synergize with rafts to provide spatiotemporal hierarchy to the membrane. Elucidating the intricate coupling of multiple spatial and temporal scales requires the application of correlative techniques, with a particular need for simultaneous nanometer spatial precision and microsecond temporal resolution. Here, we review novel fluorescence-based techniques that readily allow to decode nanoscale membrane dynamics with unprecedented spatiotemporal resolution and single-molecule sensitivity. Notably, we introduce a versatile planar nanoantenna platform combined with fluorescence correlation spectroscopy to study spatiotemporal heterogeneities on living cell membranes at the nano- up to the mesoscale. Finally, we outline remaining future technological challenges and comment on potential directions to advance our understanding of cell membrane dynamics under the influence of the actin cytoskeleton and extracellular matrix in uttermost detail.

Dynamic compartmentalization, a prominent membrane organizing principle

The concept of compartmentalization is ubiquitous in biology allowing for the complexity and function of living systems [1–6]. Fluid lipid membranes are semi-permeable barriers compartmentalizing the cell from the extracellular space and separating the intracellular space into membrane-bound organelles. However, physical and/or biochemical dynamic compartmentalization also occurs within the plane of the membrane facilitating functional, lateral subdomains. The most widely studied subdomains of biological membranes are so-called ‘membrane rafts’. According to the lipid raft hypothesis [7], such rafts are dynamic domains enriched with cholesterol, sphingolipids and other saturated lipids. These domains form tightly packed, more ordered assemblies, coexisting within the less tightly packed, non-raft membrane regions. Their function can be described as a dynamic platform for lateral protein sorting which is capable of facilitating or inhibiting interactions with other biomolecules [8].

On living cell membranes, rafts are thought to be essential for various physiological roles, e.g. signaling and membrane trafficking [1,6,9,10], although their relevance and even existence still remains disputed [1,10–16].

In model lipid membranes sterols and saturated lipids associate to a liquid ordered (Lo) phase forming domains of macroscopic as well as of nanoscopic sizes [2,17,18]. These Lo domains coexist within the liquid disordered (Ld) regions which exhibit faster lipid diffusion and flexibility. The coexistence of assemblies of order- and disorder-prefering lipids appears to dictate the membrane
organization in living cells, starting from the nanoscale and contributing to its hierarchical organization [6,19]. Domains of the Ld phase containing highly polyunsaturated and/or very short lipids increase the contrast with thicker, more tightly packed domains of the Lo phase and thus stabilize phase separation [20]. This mechanism was shown to govern the lipid membrane organization in simulations [21,22], on mimetic membranes [23,24] and on isolated cell membranes [25–27]. Studies on giant plasma membrane vesicles provided compelling evidence that the coexisting Lo-Ld phases constitute the basis for sorting membrane components in a self-organizing manner [20,28,29]. In particular, it has been reported that saturated lipids, sterols, glycolipids and certain proteins preferentially associate to more ordered phases, separated from unsaturated lipids and most other proteins [20,28,30].

Until now, several local organizers that dynamically (re)organize in time, and are responsible for the plasma membrane compartmentalization have been investigated. First, and as mentioned above, the lipid and cholesterol content of the membrane is crucial for its lateral organization. Second, proteins play an essential role in regulating, localizing, and templating the lipid environment [6–8,15,31]. Third, most likely the spatiotemporal organization of the cell membrane follows a hierarchical order from the meso-down to the nanoscale, being modulated by the cortical actin cytoskeleton and the extracellular matrix [6,9,19]. Taking together all the current insights, the cell membrane appears to be highly heterogeneous with multiple components actively and passively interacting with each other at multiple spatiotemporal scales (Figure 1A). It is also suspected that its organization serves different functions depending on the length scale [19,32].

Figure 1. The key local organizers compartmentalizing the plasma membrane and their corresponding diffusion behavior.

(A) Sketch of the heterogeneous cell membrane composed of different lipids, proteins and other molecules. Dynamic compartmentalization occurs at different spatiotemporal scales originating from lipid rafts, interactions with the extracellular matrix and glycocalyx from the outside, and also patterned by the cortical actin cytoskeleton on the cell inside. (B) To probe the spatiotemporal cell organization of the membrane, lipids and/or proteins of interest can be fluorescently tagged and their diffusion over time can be recorded. Depending on their location and time point, the diffusion behavior may be freely random, confined by the occurrence of compartments or hindered by their partitioning into domains.
lipids and proteins will exhibit a characteristic diffusion behavior which can be used as a readout for their local organization and/or interaction with other partners (Figure 1B).

**First insights into membrane dynamics with diffraction-limited optical techniques**

Over the past decades, biologists have obtained impressive insights into the complexity of living cell membranes by applying diffraction-limited optical techniques such as epi-fluorescence microscopy, confocal microscopy or Foerster resonance energy transfer (FRET) [5,28,33] combined with dynamic approaches (fluorescence correlation spectroscopy (FCS) [34–36] or fluorescence recovery after photobleaching (FRAP) [37–39]). These fluorescence-based techniques have been exploited due to their simple implementation, moderate to low phototoxicity, and high temporal resolution. However, their spatial resolution is limited by the diffraction limit of light to 200–350 nm, preventing the visualization and study of membrane compartments and domains at smaller spatial scales.

**Super-resolution fluorescence microscopy approaches to resolve nanoscale membrane dynamics**

In recent years, a wealth of fluorescence-based techniques has emerged that readily allow the study of the cell membrane with unprecedented levels of spatial and temporal details. The focus of this review is to offer a glimpse into these novel techniques, with a particular focus on the field of nanophotonics (Figure 2, acronyms included in the caption).

Super-resolution fluorescence microscopy overcomes the diffraction limit of light by exploiting the photophysical properties of labeling fluorophores together with tailored illumination schemes. As such, they have provided insights into nanoscale regions of the plasma membrane, mainly of fixed cells, with much greater spatial resolution. Super-resolution techniques based on single-molecular localization methods (SMLM) have resolved receptor nanoclusters on the cell membrane in the range of a few nm, routinely of 10–30 nm [41,42]. A highly advancing approach are SMLM implementations relying on DNA-based probes (i.e. DNA-PAINT) instead of conventional fluorophores which allow for quantitative studies at ultra-high spatial resolutions and in a multicolor fashion [43,44]. Unfortunately, so far, SMLM remain being too slow for true applications in living cells, in particular for cell membrane studies.

Stimulated emission depletion (STED) microscopy is another powerful super-resolution approach, which continues to push the diffraction limit of light [45]. STED relies on engineering two focused laser beams to exclusively detect the fluorescence within a small, nanometric focal spot while depleting the peripheral fluorescence via stimulated emission. In view of biological applications, the combination of STED nanoscopy with FCS has been a major milestone [46–49]. STED-FCS resolved the transient trapping (<10–20 ms) of sphingolipids and GPI-anchored proteins (GPI-APs) into cholesterol-dependent nanoscopic domains as small as 30 nm in size [47]. The advent of fast laser-beam scanners made it possible to record multiple FCS measurements by scanning along a line or circle at the micron-scale with kHz frequency, i.e. sSTED-FCS [50]. This approach allowed to investigate dynamics occurring on living cell membranes with ~60 nm spatial resolution. Successively, STED-FCS implementations employing fluorescence lifetime gating have been accomplished on living cells with improved resolution (~40 nm) and lower depletion power, thus less phototoxicity [49,51]. However, the tradeoff between the photon budget and technical constraints has not yet allowed to breach through ~40 nm of spatial and millisecond temporal resolution simultaneously [49,50,52]. Recently, MINFLUX nanoscopy has demonstrated 1–3 nm resolution for structures in fixed and living cells. MINFLUX nanoscopy is neatly merging different super-resolution approaches as it relies on localizing single switchable fluorophores by applying a donut-shaped excitation beam similar to that provided by STED [40,53]. On model membranes MINFLUX was able to follow the diffusion of single DPPE fluorescent lipid analogs with kHz count rate at <20 nm localization and ~100 µs sampling time [54]. Note that this was achieved in a sparsely fluorescently labeled environment, an important drawback of the technique that still needs to be overcome. Single-particle tracking (SPT) is a powerful technique to track individual molecules as they diffuse on the plasma membrane with nanometer localization precision. Since the initial SPT implementation to uncover live-cell dynamics [4,55,56], SPT methods continuously improved with regard to camera sampling speed, fluorescent labeling strategies and tracking algorithms [57,58]. SPT has provided enormous information on the nanoscale dynamics of raft-associated GPI-APs on the cell membrane. All studied GPI-APs appear to continuously assemble in
transient (~200 ms) homodimers likely constituting a possible basic unit of raft domains (~3–15 nm in size) depending on dimer-lipid interactions [31]. Subsequently, using dual-color SPT and improved temporal resolution (down to 0.5 ms) it was shown that gangliosides dynamically interact on the timescale of ~10 ms with monomers and dimers of GPI-AP in a cholesterol-dependent manner [59]. Also with live-cell SPT the rapid exchange of sphingomyelin (SM) between GPI-AP assemblies and the bulk membrane has been shown [60]. These results (and others reported earlier) endorse the dynamic character of membrane rafts composed of SM, cholesterol, and raft-associated proteins in constant exchange with the bulk membrane at the millisecond to second timescale.

Recently, live-cell SPT has been coupled to photo-activated localization microscopy (PALM), i.e. sptPALM, enabling the visualization of diffusion dynamics of single molecules at high labeling densities while maintaining nanometer localization precision. The approach permitted to generate high-density single-molecule maps of the
Gag and VSVG membrane proteins showing different diffusion and clustering behavior [61]. This technique also resolved the interaction dynamics between membrane and water-soluble proteins on the crowded living cell membrane [62]. Furthermore, the nanoscale organization of integrins within focal adhesions has been elucidated with ∼50 nm spatial resolution on the living cell membrane [63]. Complementing sptPalm with molecule dynamics simulations revealed the spatiotemporal dynamics of the Ras protein and in conjunction with a possible explanation of its role in nanodomain formation and signaling [64,65]. These insights could potentially be generalized to decode the nanoscale dynamics of more membrane molecules.

Interferometric scattering (iSCAT) microscopy allows to track single-molecule trajectories at video sampling rates by using 20–40 nm gold nanoparticles as labeling probes [66]. Recently, iSCAT together with extensive image analysis was able to track the dynamics of an unlabeled protein with nm spatial and μs temporal resolution in 3D over tens of minutes [67]. These results highlight the potential of iSCAT for quantitative live-cell studies in future.

**Correlative nanophotonic approaches offering ultrahigh spatiotemporal resolution**

A different approach to explore nanometric regions of the membrane with increased temporal resolution relies on the booming field of nanophotonics. Nanophotonic approaches are based on metallic (plasmonic) nanostructures, also termed as plasmonic or photonic nanoantennas, that localize and enormously enhance the excitation field into nanometric regions (plasmonic hotspots) [68]. Hereby, the incident light does not further propagate but remains highly localized in the near-field proximity of the nanoantenna. The huge potential of photonic nanoantennas for biological applications lies in the following two properties. First, nanoantennas provide highly enhanced electromagnetic near-fields resulting in high fluorescent enhancement of fluorescent molecules having matching absorption and/or emission spectra to that of the antenna resonance. Second, nanoantennas offer highly localized hotspots of illumination, thus drastically decreasing the observation volume.

The simplest, yet powerful nanophotonic structure consists in nanometric apertures (typically of 50–200 nm radii) which are either fabricated onto a planar surface, so-called zero mode waveguides (ZMWs) (Figure 3A) or onto the apex of an optical fiber which is then mounted on a scanning microscope, the near-field scanning microscopy (NSOM) approach (Figure 3B, left). Due to the subwavelength size of the nanoaperture, an exponentially decaying electromagnetic field is sustained acting as an effective pinhole upon illumination. Such a near-field profile as afforded by ZMW and NSOM provides effective detection volumes which are three orders of magnitude below the diffraction-limited spot and enable single-molecule detection at micromolar concentrations [69].

ZMWs and aperture-based NSOM have been combined with FCS and were successfully applied to follow living cell membrane dynamics. For instance, the partitioning of ganglioside proteins into 30 nm membrane domains has been revealed by means of ZMWs [70]. Filling the nanoapertures with fused silica led to planarized ZMWs preventing invaginations in living cell membrane studies [71]. The implementation of ZMWs in arrays together with wide field detection has further enabled parallelized readout over many ZMW simultaneously. Impressive results have been reported on real-time DNA sequencing [72], living cell membranes [73] and single-molecule FRET of a microfluidic chip [74]. In the case of aperture-based NSOM together with FCS, it was possible to uncover the anomalous diffusion behavior of the fluorescent lipid analog SM in living cells within regions of ∼100 nm in size [75]. An exclusive advantage of NSOM over ZMWs is the possibility of performing fluorescence imaging with nanometric resolution together with simultaneous topographic recording of the cell surface. This approach has revealed nanoclustering of different immune receptors on the plasma membrane [76–78] and allowed the visualization of small rafts enriched by GPI-APs and gangliosides on intact fixed cell membranes [78,79]. The main limitation of conventional nanoapertures as implemented in ZMWs and NSOM is the low light throughput exiting from these structures as they do not provide field enhancement. As such, exploiting these nanoapertures for the study of processes below 50 nm becomes highly challenging.

To overcome this limitation, a leap forward for the NSOM configuration was achieved by engineering a monopole antenna at the edge of the NSOM aperture (Figure 3B, right) [80]. Using this configuration, the authors could not only show increased spatial resolution but, importantly, they demonstrated that nanoantennas can manipulate the directional emission of individual molecules. This type of monopole antenna has been used to image individual proteins and receptor nanodomains on intact cell membranes with an unprecedented
spatial resolution of $\sim 30$ nm [81]. More recent designs include bowtie antenna apertures (Figure 3C, left) and hybrid antennas combining a monopole with a bowtie (Figure 3C, right) [82,83]. This hybrid configuration reached true spatial resolution of 20 nm together with sub-nm localization accuracy on individual molecules and in a multicolor fashion [83] and has been exploited more recently to control the degree of FRET on single DNA strands [84].

Similar types of nanoantennas have been directly fabricated on planar surfaces (Figure 3D). With such planar antenna designs light confinement into sub-20 nm hotspots has been successfully demonstrated [85–87]. Moreover, enhancement factors of over thousand-fold for single-molecule fluorescence signals were achieved with gold bowtie antennas [85], DNA-origami gold dimers [86,88], and at the apex of gold nanorods [89–95]. In addition, single molecule tracking on supported membranes has been demonstrated using arrays of optical nanoantennas [96]. These exciting results underscore the potential of nanoantennas for multiple applications, including biosensing and live-cell studies.

Plasmonic biosensing has grown into a highly active and innovative field on its own as excellently summarized in other reviews [97–100]. Notable plasmonic biosensing applications in the context of nanomedicine are enhanced Raman spectroscopy with high throughput on microfluidic devices with single-cell sensitivity [101] and the detection of single amino acid mutations in breast cancer cells [102]. Current efforts emphasize the need for nanofabrication approaches to engineer large-scale low-cost platforms for high-throughput label-free detection. Two versatile platforms of large-scale antenna arrays based on gold nanoholes [103] or nanogap
antennas fabricated by hole-mask colloidal lithography [104] reported high sensitivity and multiplexing capacities for point-of-care applications.

To extend the application of nanoantennas to live-cell studies, they need to provide efficient rejection of the surrounding background fluorescence and accessible illumination hotspots on a planar, biocompatible substrate at large scale. A reliable large-scale nanoantenna fabrication has been achieved and validated on living cell membranes using bowtie nanoantennas of reproducible 20 nm gaps (Figure 3E) [105]. Successful fluorescent background reductions were initially based on exploiting weak emitters (quantum yield <8%) which allow to obtain high fluorescence enhancement within antenna hotspots while keeping the background signal to a minimum [85,88,91–93,105–108]. Another initial attempt was based on lifetime filtering [89,109,110] since the presence of an antenna dramatically reduces the fluorescence lifetime of molecules [111].

Planar ‘antenna-in-box’ platform to resolve live-cell membrane dynamics

To allow for fluorescence experiments with single-molecule sensitivity at physiologically relevant concentrations an innovative ‘antenna-in-box’ design has been developed [87]. This design consists of a dimer nanogap antenna made of gold and centered inside a nanoaperture (Figure 3F). The nanogap determines the nanoscale confinement of the incoming light into a plasmonic hotspot whereas the surrounding metallic cladding efficiently screens the fluorescence background. The final improvement to achieve a planar ‘antenna-in-box’ platform was attained by adding template stripping to the multistep nanofabrication process [112]. This led to the readily availability of a planar platform containing thousands of narrow nanogap antennas with accessible plasmonic hotspots compatible with dynamic live-cell studies. This planar nanoantenna platform combined with FCS was applied to follow the nanoscale lipid dynamics on mimetic as well as on living cell membranes [18,113]. Together with cholesterol-depletion experiments, compelling evidence of cholesterol-induced ~10 nm nanodomains with ∼0.9 ms transient trapping times partitioning on the living membrane was reported [113]. These results confirmed the existence of highly dynamic raft nanodomains on the living cell membrane. Moreover, this work validates the potential of planar plasmonic antenna arrays combined with fluorescence microscopy to enlighten and quantify the dynamics and interactions of lipids and raft-associated proteins on the living cell membrane.

The large-scale availability of planar nanogap antenna arrays facilitate their implementation for live-cell imaging including the adaptation of established biological sample preparation protocols. Additionally, these types of platforms accommodate gap sizes between 10 to 50 nm which have been exploited to perform so-called ‘spot-variation FCS’ [114] at the nanoscale [18,113,115,116], at scales significantly smaller than possible with sSTED-FCS [50] or ZMWs [117,118]. Moreover, a planar nanoantenna platform can be readily combined with other techniques to increase the information content of the sample under study. For instance, correlative studies of antenna-FCS and atomic force microscopy (AFM) and spectroscopy permitted to resolve the influence of hyaluronic acid, an abundant ECM component, on the nanoscale lipid organization of model lipid membranes [115]. This shows the potential to use the planar nanogap antennas for correlative measurements, albeit requiring substrate optimization. The fixed antenna positions on the planar substrate enable measurements under different (treatment) conditions on the exact same position and/or to correlate measured diffusion behavior with spatial location. Since the antenna platform provides diffraction-limited spots (nanoaperture without antenna) on the same substrate, the nanoscale diffusion behavior can be directly linked with that obtained by confocal means.

In sum, the planar nanogap antenna arrays comprise a versatile platform to study spatiotemporal heterogeneities on living cell membranes at the nano- up to the mesoscale. Future directions involve the multi-color extension of current antenna approaches to examine nanoscale interactions between different molecules, including lipids and proteins. This can be achieved by choosing the right antenna material, for instance replacing gold by aluminum [105,118,119] or by the use of novel (dielectric) materials [120–122]. Another future challenge consists in increasing the throughput of experiments by enabling parallel detection of hundreds of antennas simultaneously, in an analogous way as to ZMWs [69,73,74]. One possible multiplexing approach would be to switch from confocal to wide-field illumination and from APDs to a fast camera detection scheme [123,124]. Although the temporal resolution is reduced to the millisecond regime by switching to camera detection, the diffusion of most proteins in the living cell membrane is also two orders of magnitude slower than freely diffusing dyes. Thus, the tradeoff between camera framerate, smaller diffusion areas and the photon budget of the
fluorescent labels should guarantee for a high signal-to-noise ratio to investigate live-cell dynamics. One additional advantage provided by antennas lies in the fluorescent enhancement of the molecules interacting with the antennas. This adds flexibility when choosing the fluorescent probe as weakly emitting dyes can equally be used, in strong contrast with STED-FCS or other SPT-based approaches that require bright and photostable dyes. However, a careful characterization of the complex antenna’s near-field profile is required to derive quantification of the experimental data and unbiased data interpretation.

Conclusion
Together, the approaches discussed in this review show capabilities to decipher the nanoscale organization and/or nanoscale diffusion dynamics on living cell membranes. Impressive insights have been achieved which have increased our understanding of the spatiotemporal cell membrane organization and its link to function. Yet, despite the collective effort of the community to develop and optimize imaging approaches offering high spatiotemporal resolution, challenges remain. Most super-resolution approaches would highly benefit from fluorescent probes with enhanced photophysical properties (brightness and photostability) and minimal sample perturbation [125,126]. Encouraging advances for improved fluorophores rely on sophisticated genetic modifications [127], biorthogonal labeling strategies and click-chemistry [128,129]. With the quest for high-throughput solutions, efficient data processing and an expanded toolbox of analysis algorithms become crucial. In this aspect, machine-learning approaches are promising to not only automatize data analysis but also to remove human bias. Besides, machine learning has been employed to create tailor-made optimized designs for nanoantenna designs [130].

We have experienced a growing pursuit to combine existing techniques leading to a multitude of correlative approaches. Successful implementations are sptPALM, STED-FCS, FCS combined with nanoantenna arrays and even with AFM. Such solutions not only multiply the wealth of information but also amplify the reliability of the obtained data. For example, a combined FCS-AFM approach permits to track the diffusion of the molecule of interest as well as to locate its position on the sample’s topography. We envision that the quest for more and improved correlative approaches will continue and will encompass molecular force measurements. To accelerate the development and expansion of the toolbox of super-resolved single-molecule techniques for live-cell studies, the close collaboration among researchers across fields will be key.

In conclusion, we provided a glimpse into the currently available techniques capable to follow nanoscale dynamics on the living cell membrane with ultrahigh spatiotemporal resolution and single-molecule sensitivity. We gave an overview of challenges to overcome and presented future directions. We highlighted the great potential of photonic nanoantennas combined with FCS to become a versatile, correlative toolbox to study live-cell dynamics spanning the nano- up to the mesoscale. Particularly, we shall witness how these advances resolve dynamics on the living cell membrane under the influence of the actin cytoskeleton and extracellular matrix in uttermost detail. So, we will eventually understand the mechanisms governing the basic unit of life, the cell.

Perspectives

- Dynamic compartmentalization is a prevailing principle regulating the spatiotemporal organization of the living cell membrane at multiple scales. Correlative nanophotonic approaches provide the required nanometer spatial and sub-millisecond temporal resolution to enlighten nanoscale live-cell membrane dynamics.

- Planar nanogap antenna arrays combined with FCS are a versatile platform to follow single-molecule diffusion in the crowded environment of the living cell membrane. The large-scale availability of planar nanoantennas with gap sizes between 10 to 50 nm have been exploited to perform ‘spot-variation FCS’ at the nanoscale reporting on transient nanoscopic heterogeneities on model lipid and on living cell membranes. The nanoantenna-FCS approach can be combined with other techniques such as AFM and force spectroscopy to provide correlative information on the plasma membrane organization.
• We envision that the currently available toolbox of super-resolved and nanophotonic techniques for live-cell studies will rapidly expand and will encompass improved fluorophores, multicolor extension, high-throughput solutions, and efficient data processing. A close collaboration among researchers across fields will be key to eventually understand the mechanisms governing the basic unit of life, the cell.

Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

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P.M.W. and M.F.G.-P. wrote the manuscript. P.M.W. prepared the figures.

Abbreviations
AFM, atomic force microscopy; FCS, fluorescence correlation spectroscopy; FRAP, fluorescence recovery after photobleaching; FRET, Förster resonance energy transfer; NSOM, near-field scanning microscopy; SM, sphingomyelin; SMLM, single-molecular localization methods; SPT, single-particle tracking; STED, stimulated emission depletion; ZMWs, zero mode waveguides.

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