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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. We particularly focus on correlative approaches from the field of nanophotonics. 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-preferring 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]. Influenced by their environment,
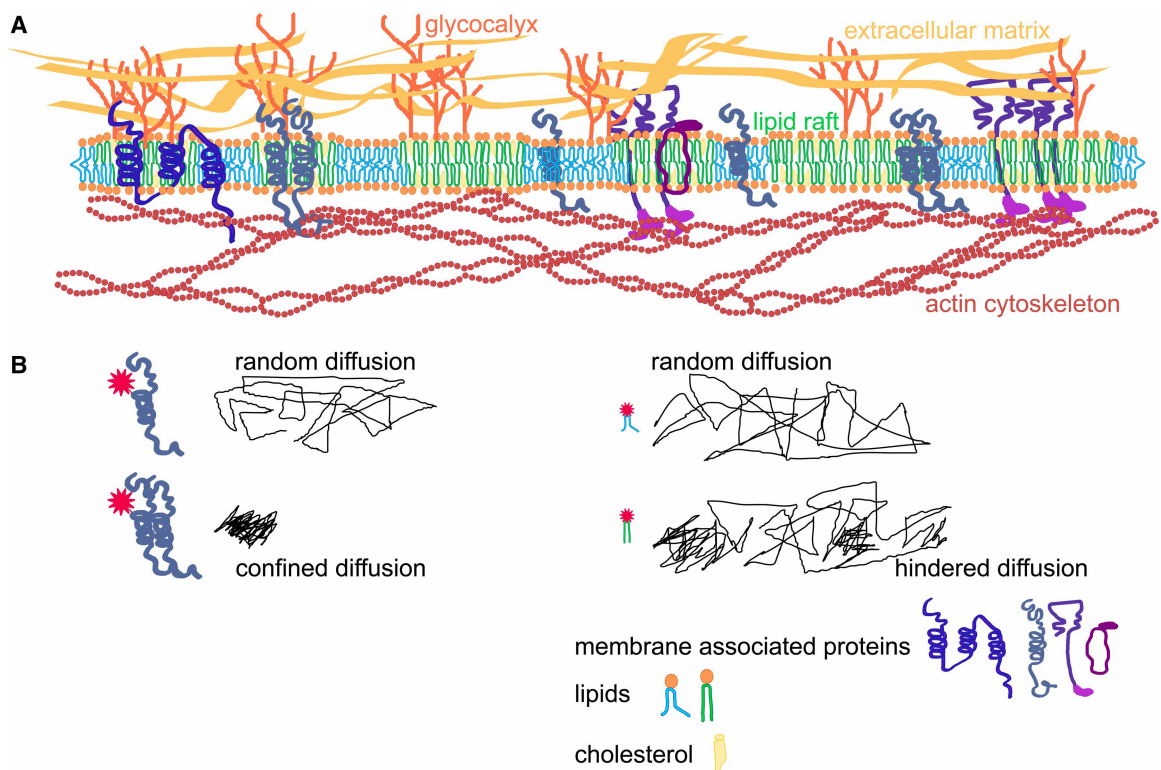
Figure 1. The key local organizers compartmentalizing the plasma membrane and their corresponding diffusion behavior.

(**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 multi-color 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
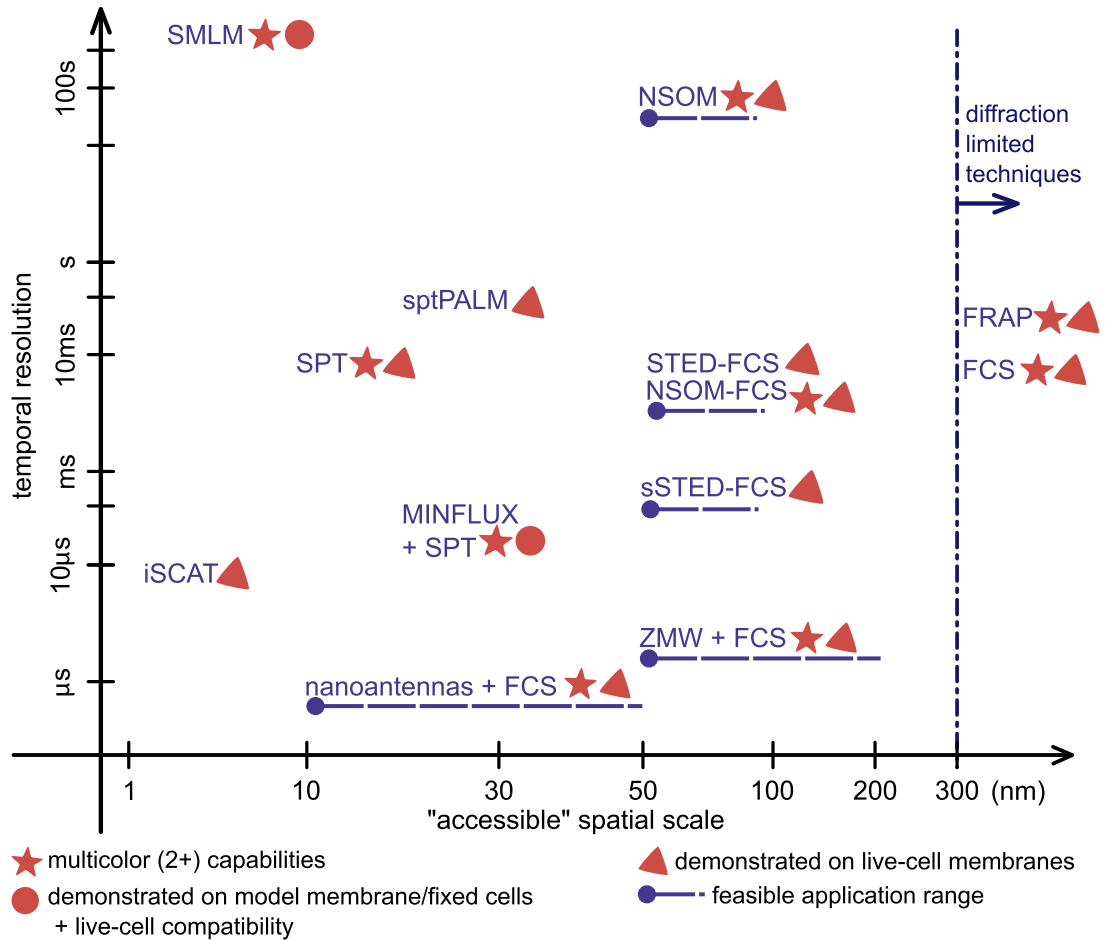
Figure 2. Super-resolution approaches for membrane dynamics studies.

Currently available super-resolution approaches are displayed with respect to the accessible spatial scale and temporal resolution. In addition, each approach is highlighted regarding to its capability for multi-color extension and whether applications have been reported on model membranes or living cell membranes. Acronyms: FCS: fluorescence correlation spectroscopy, FRAP: fluorescence recovery after photobleaching, SMLM: single-molecule localization microscopy, NSOM: near-field scanning optical microscopy, SPT: single particle tracking, sptPALM: single particle tracking photo-activated localization microscopy, (s)STED: (scanning)stimulated emission microscopy, iSCAT: interferometric scattering microscopy, ZMW: zero-mode waveguide. Note that MINFLUX is not an acronym but 'a concept for localizing photon emitters in space' [40]. Also note that we excluded super-resolution approaches such as structured illumination microscopy (SIM) because the main applications are for 3D-live cell imaging rather than cell membrane studies [41], and other computational approaches such as SRRF, SOFI, 3B, HAWK, etc.

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
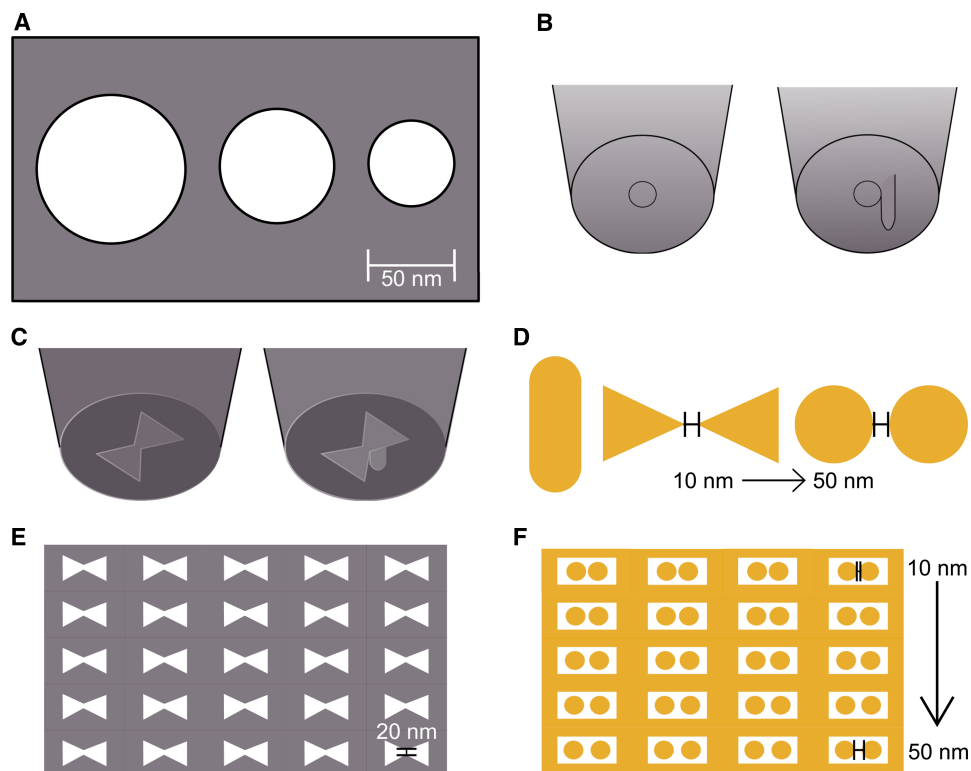
Figure 3. Exemplary nanophotonic designs.
(**A**) Zero-mode-waveguides (ZMW) of different radii fabricated into an aluminum film. (**B**) The initial NSOM approach consisting in a circular nanoaperture (left) and the integration of a monopole antenna to enhance the light throughput and to provide ultra-high field confinement. (**C**) NSOM-antenna realizations by replacing the circular nanoaperture by a bowtie antenna (left) and by combining the bowtie aperture with a monopole antenna (right) leading to a hybrid nanoantenna. (**D**) Nanoantenna configurations in the shape of a nanorod, bowtie or dimer fabricated onto a planar substrate. (**E**) Large-scale implementation of bowtie nanoantenna arrays on a planar surface. (**F**) Planar 'antenna-in-box' platform consisting of arrays of dimer antennas embedded in a nanoaperture. Arrays of antennas can be routinely fabricated with different gap sizes, from 50 nm down to 10 nm in size.

spatial resolution of ∼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.

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- 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, multi-color 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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Author Contributions

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, Foerster 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.

References

1   Lingwood, D. and Simons, K. (2010) Lipid rafts as a membrane-organizing principle. *Science* **327**, 46–50 https://doi.org/10.1126/science.1174621
2   Brown, D.A. and London, E. (1998) Functions of lipid rafts in biological membranes. *Annu. Rev. Cell Dev. Biol.* **14**, 111–136 https://doi.org/10.1146/annurev.cellbio.14.1.111
3   Brown, D.A. and London, E. (2000) Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *J. Biol. Chem.* **275**, 17221–17224 https://doi.org/10.1074/jbc.R000005200
4   Kusumi, A., Nakada, C., Ritchie, K., Murase, K., Suzuki, K., Murakoshi, H. et al. (2005) Paradigm shift of the plasma membrane concept from the two-dimensional continuum fluid to the partitioned fluid: high-speed single-molecule tracking of membrane molecules. *Annu. Rev. Biophys. Biomol. Struct.* **34**, 351–378 https://doi.org/10.1146/annurev.biophys.34.040204.144637
5   Gowrishankar, K., Ghosh, S., Saha S, C.R., Mayor, S. and Rao, M. (2012) Active remodeling of cortical actin regulates spatiotemporal organization of cell surface molecules. *Cell* **149**, 1353–1367 https://doi.org/10.1016/j.cell.2012.05.008
6   Sezgin, E., Levental, I., Mayor, S. and Eggeling, C. (2017) The mystery of membrane organization: composition, regulation and roles of lipid rafts. *Nat. Rev. Mol. Cell Biol.* **18**, 361–374 https://doi.org/10.1038/nrm.2017.16
7   Simons, K. and Ikonen, E. (1997) Functional rafts in cell membranes. *Nature* **387**, 569 https://doi.org/10.1038/42408
8   Stone, M.B., Shelby, S.A., Núñez, M.F., Wisser, K. and Veatch, S.L. (2017) Protein sorting by lipid phase-like domains supports emergent signaling function in B lymphocyte plasma membranes. *eLife* **6**, e19891 https://doi.org/10.7554/eLife.19891
9   Garcia-Parajo, M.F., Cambi, A., Torreno-Pina, J.A., Thompson, N. and Jacobson, K. (2014) Nanoclustering as a dominant feature of plasma membrane organization. *J. Cell Sci.* **127**, 4995–5005 https://doi.org/10.1242/jcs.146340
10 Mayor, S. and Rao, M. (2004) Rafts: scale-dependent, active lipid organization at the cell surface: raft hypothesis. *Traffic* **5**, 231–240 https://doi.org/10.1111/j.1600-0854.2004.00172.x
11 Munro, S. (2003) Lipid rafts: elusive or illusive? *Cell* **115**, 377–388 https://doi.org/10.1016/S0092-8674(03)00882-1
12 Brown, D.A. (2001) Seeing is believing: visualization of rafts in model membranes. *Proc. Natl Acad. Sci. U.S.A.* **98**, 10517–10518 https://doi.org/10.1073/pnas.191386898
13 Hancock, J.F. (2006) Lipid rafts: contentious only from simplistic standpoints. *Nat. Rev. Mol. Cell Biol.* **7**, 456–462 https://doi.org/10.1038/nrm1925
14 Pike, L.J. (2006) Rafts defined: a report on the keystone symposium on lipid rafts and cell function. *J. Lipid Res.* **47**, 1597–1598 https://doi.org/10.1194/jlr.E600002-JLR200
15 Simons, K. and Gerl, M.J. (2010) Revitalizing membrane rafts: new tools and insights. *Nat. Rev. Mol. Cell Biol.* **11**, 688–699 https://doi.org/10.1038/nrm2977

16   Jacobson, K., Mouritsen, O.G. and Anderson, R.G.W. (2007) Lipid rafts: at a crossroad between cell biology and physics. *Nat. Cell Biol.* **9**, 7–14 https://doi.org/10.1038/ncb0107-7

17   Dietrich, C., Bagatolli, L.A., Volovyk, Z.N., Thompson, N.L., Levi, M., Jacobson, K. et al. (2001) Lipid rafts reconstituted in model membranes. *Biophys. J.* **80**, 1417–1428 https://doi.org/10.1016/S0006-3495(01)76114-0

18   Winkler, P.M., Regmi, R., Flauraud, V., Brugger, J., Rigneault, H., Wenger, J. et al. (2017) Transient nanoscopic phase separation in biological lipid membranes resolved by planar plasmonic antennas. *ACS Nano* **11**, 7241–7250 https://doi.org/10.1021/acsnano.7b03177

19   Kalappurakkal, J.M., Sil, P. and Mayor, S. (2020) Toward a new picture of the living plasma membrane. *Protein Sci.* **29**, 1355–1365 https://doi.org/10.1002/pro.3874

20   Levental, I., Grzybek, M. and Simons, K. (2011) Raft domains of variable properties and compositions in plasma membrane vesicles. *Proc. Natl Acad. Sci. U.S.A.* **108**, 11411–11416 https://doi.org/10.1073/pnas.1105996108

21   Lin, X., Lorent, J.H., Skinkle, A.D., Levental, K.R., Waxham, M.N., Gorfe, A.A. et al. (2016) Domain stability in biomimetic membranes driven by lipid polyunsaturation. *J. Phys. Chem. B* **120**, 11930–11941 https://doi.org/10.1021/acs.jpcb.6b06815

22   Janosi, L., Li, Z., Hancock, J.F. and Gorfe, A.A. (2012) Organization, dynamics, and segregation of Ras nanoclusters in membrane domains. *Proc. Natl Acad. Sci. U.S.A.* **109**, 8097–8102 https://doi.org/10.1073/pnas.1200773109

23   Heberle, F.A., Petruzielo, R.S., Pan, J., Drazba, P., Kučerka, N., Standaert, R.F. et al. (2013) Bilayer thickness mismatch controls domain size in model membranes. *J. Am. Chem. Soc.* **135**, 6853–6859 https://doi.org/10.1021/ja3113615

24   Nyholm, T.K.M., Lindroos, D., Westerlund, B. and Slotte, J.P. (2011) Construction of a DOPC/PSM/Cholesterol phase diagram based on the fluorescence properties of *trans*-parinaric acid. *Langmuir* **27**, 8339–8350 https://doi.org/10.1021/la201427w

25   Sezgin, E., Levental, I., Grzybek, M., Schwarzmann, G., Mueller, V., Honigmann, A. et al. (2012) Partitioning, diffusion, and ligand binding of raft lipid analogs in model and cellular plasma membranes. *Biochim. Biophys. Acta Biomembr.* **1818**, 1777–1784 https://doi.org/10.1016/j.bbamem.2012.03.007

26   Sezgin, E., Gutmann, T., Buhl, T., Dirkx, R., Grzybek, M., Coskun, Ü. et al. (2015) Adaptive lipid packing and bioactivity in membrane domains. *PLoS ONE* **10**, e0123930 https://doi.org/10.1371/journal.pone.0123930

27   Levental, K.R., Lorent, J.H., Lin, X., Skinkle, A.D., Surma, M.A., Stockenbojer, E.A. et al. (2016) Polyunsaturated lipids regulate membrane domain stability by tuning membrane order. *Biophys. J.* **110**, 1800–1810 https://doi.org/10.1016/j.bpj.2016.03.012

28   Baumgart, T., Hammond, A.T., Sengupta, P., Hess, S.T., Holowka, D.A., Baird, B.A. et al. (2007) Large-scale fluid/fluid phase separation of proteins and lipids in giant plasma membrane vesicles. *Proc. Natl Acad. Sci. U.S.A.* **104**, 3165–3170 https://doi.org/10.1073/pnas.0611357104

29   Lingwood, D., Ries, J., Schwille, P. and Simons, K. (2008) Plasma membranes are poised for activation of raft phase coalescence at physiological temperature. *Proc. Natl Acad. Sci. U.S.A.* **105**, 10005–10010 https://doi.org/10.1073/pnas.0804374105

30   Levental, K.R. and Levental, I. (2015) Giant plasma membrane vesicles: models for understanding membrane organization. *Curr. Top. Membr.* **75**, 25–57 https://doi.org/10.1016/bs.ctm.2015.03.009

31   Suzuki, K.G.N., Kasai, R.S., Hirosawa, K.M., Nemoto, Y.L., Ishibashi, M., Miwa, Y. et al. (2012) Transient GPI-anchored protein homodimers are units for raft organization and function. *Nat. Chem. Biol.* **8**, 774–783 https://doi.org/10.1038/nchembio.1028

32   Levental, I., Levental, K.R. and Heberle, F.A. (2020) Lipid rafts: controversies resolved, mysteries remain. *Trends Cell Biol.* **30**, 341–353 https://doi.org/10.1016/j.tcb.2020.01.009

33   Goswami, D., Gowrishankar, K., Bilgrami, S., Ghosh, S., Raghupathy, R., Chadda, R. et al. (2008) Nanoclusters of GPI-anchored proteins Are formed by cortical actin-driven activity. *Cell* **135**, 1085–1097 https://doi.org/10.1016/j.cell.2008.11.032

34   Marguet, D., Lenne, P., Rigneault, H. and He, H. (2006) Dynamics in the plasma membrane: how to combine fluidity and order. *EMBO J.* **25**, 3446 https://doi.org/10.1038/sj.emboj.7601204

35   Bacia, K., Kim, S.A. and Schwille, P. (2006) Fluorescence cross-correlation spectroscopy in living cells. *Nat. Methods* **3**, 83–89 https://doi.org/10.1038/nmeth822

36   He, H.-T. and Marguet, D. (2011) Detecting nanodomains in living cell membrane by fluorescence correlation spectroscopy. *Annu. Rev. Phys. Chem.* **62**, 417–436 https://doi.org/10.1146/annurev-physchem-032210-103402

37   Meder, D., Moreno, M.J., Verkade, P., Vaz, W.L.C. and Simons, K. (2006) Phase coexistence and connectivity in the apical membrane of polarized epithelial cells. *Proc. Natl Acad. Sci. U.S.A.* **103**, 329–334 https://doi.org/10.1073/pnas.0509885103

38   Kenworthy, A.K. (2007) Fluorescence Recovery After Photobleaching Studies of Lipid Rafts. In *Lipid Rafts* (McIntosh, T.J., ed.), pp. 179–192, Humana Press, Totowa, NJ

39   Chen, Y., Lagerholm, B.C., Yang, B. and Jacobson, K. (2006) Methods to measure the lateral diffusion of membrane lipids and proteins. *Methods* **39**, 147–153 https://doi.org/10.1016/j.ymeth.2006.05.008

40   Balzarotti, F., Eilers, Y., Gwosch, K.C., Gynnå, A.H., Westphal, V., Stefani, F.D. et al. (2017) Nanometer resolution imaging and tracking of fluorescent molecules with minimal photon fluxes. *Science* **355**, 606–612 https://doi.org/10.1126/science.aak9913

41   Sherman, E., Barr, V., Manley, S., Patterson, G., Balagopalan, L., Akpan, I. et al. (2011) Functional nanoscale organization of signaling molecules downstream of the T cell antigen receptor. *Immunity* **35**, 705–720 https://doi.org/10.1016/j.immuni.2011.10.004

42   Pageon, S.V., Tabarin, T., Yamamoto, Y., Ma, Y., Nicovich, P.R., Bridgeman, J.S. et al. (2016) Functional role of T-cell receptor nanoclusters in signal initiation and antigen discrimination. *Proc. Natl Acad. Sci. U.S.A.* **113**, E5454–E5463 https://doi.org/10.1073/pnas.1607436113

43   Schnitzbauer, J., Strauss, M.T., Schlichthaerle, T., Schueder, F. and Jungmann, R. (2017) Super-resolution microscopy with DNA-PAINT. *Nat. Protoc.* **12**, 1198–1228 https://doi.org/10.1038/nprot.2017.024

44   Nieves, D., Gaus, K. and Baker, M. (2018) DNA-based Super-resolution microscopy: DNA-PAINT. *Genes* **9**, 621 https://doi.org/10.3390/genes9120621

45   Kastrup, L., Blom, H., Eggeling, C. and Hell, S.W. (2005) Fluorescence fluctuation spectroscopy in subdiffraction focal volumes. *Phys. Rev. Lett.* **94**, 178104 https://doi.org/10.1103/PhysRevLett.94.178104

46   Saka, S.K., Honigmann, A., Eggeling, C., Hell, S.W., Lang, T. and Rizzoli, S.O. (2014) Multi-protein assemblies underlie the mesoscale organization of the plasma membrane. *Nat. Commun.* **5**, 4509 https://doi.org/10.1038/ncomms5509

47   Eggeling, C., Ringemann, C., Medda, R., Schwarzmann, G., Sandhoff, K., Polyakova, S. et al. (2009) Direct observation of the nanoscale dynamics of membrane lipids in a living cell. *Nature* **457**, 1159–1162 https://doi.org/10.1038/nature07596

48 Mueller, V., Ringemann, C., Honigmann, A., Schwarzmann, G., Medda, R., Leutenegger, M. et al. (2011) STED nanoscopy reveals molecular details of cholesterol- and cytoskeleton-modulated lipid interactions in living cells. *Biophys. J.* **101**, 1651–1660 https://doi.org/10.1016/j.bpj.2011.09.006

49 Vicidomini, G., Ta, H., Honigmann, A., Mueller, V., Clausen, M.P., Waithe, D. et al. (2015) STED-FLCS: an advanced tool to reveal spatiotemporal heterogeneity of molecular membrane dynamics. *Nano Lett.* **15**, 5912–5918 https://doi.org/10.1021/acs.nanolett.5b02001

50 Honigmann, A., Mueller, V., Ta, H., Schoenle, A., Sezgin, E., Hell, S.W. et al. (2014) Scanning STED-FCS reveals spatiotemporal heterogeneity of lipid interaction in the plasma membrane of living cells. *Nat. Commun.* **5**, 5412 https://doi.org/10.1038/ncomms6412

51 Vicidomini, G., Moneron, G., Han, K.Y., Westphal, V., Ta, H., Reuss, M. et al. (2011) Sharper low-power STED nanoscopy by time gating. *Nat. Methods* **8**, 571–573 https://doi.org/10.1038/nmeth.1624

52 Göttfert, F., Pleiner, T., Heine, J., Westphal, V., Görlich, D., Sahl, S.J. et al. (2017) Strong signal increase in STED fluorescence microscopy by imaging regions of subdiffraction extent. *Proc. Natl Acad. Sci. U.S.A.* **114**, 2125–2130 https://doi.org/10.1073/pnas.1621495114

53 Gwosch, K.C., Pape, J.K., Balzarotti, F., Hoess, P., Ellenberg, J., Ries, J. et al. (2020) MINFLUX nanoscopy delivers 3D multicolor nanometer resolution in cells. *Nat. Methods* **17**, 217–224 https://doi.org/10.1038/s41592-019-0688-0

54 Schmidt, R., Weihs, T., Wurm, C.A., Jansen, I., Rehman, J., Sahl, S.J. et al. (2021) MINFLUX nanometer-scale 3D imaging and microsecond-range tracking on a common fluorescence microscope. *Nat. Commun.* **12**, 1478 https://doi.org/10.1038/s41467-021-21652-z

55 Saxton, M.J. and Jacobson, K. (1997) SINGLE-PARTICLE TRACKING: applications to membrane dynamics. *Annu Rev Biophys. Biomol. Struct.* **26**, 373–399 https://doi.org/10.1146/annurev.biophys.26.1.373

56 Dietrich, C., Yang, B., Fujiwara, T. and Kusumi, A.) Jacobson K relationship of lipid rafts to transient confinement zones detected by single particle tracking. *Biophys. J.* **82**, 274–284 https://doi.org/10.1016/S0006-3495(02)75393-9

57 Manzo, C. and Garcia-Parajo, M.F. (2015) A review of progress in single particle tracking: from methods to biophysical insights. *Rep. Prog. Phys.* **78**, 124601 https://doi.org/10.1088/0034-4885/78/12/124601

58 Li, N., Zhao, R., Sun, Y., Ye, Z., He, K. and Fang, X. (2017) Single-molecule imaging and tracking of molecular dynamics in living cells. *Natl Sci. Rev.* **4**, 739–760 https://doi.org/10.1093/nsr/nww055

59 Komura, N., Suzuki, K.G.N., Ando, H., Konishi, M., Koikeda, M., Imamura, A. et al. (2016) Raft-based interactions of gangliosides with a GPI-anchored receptor. *Nat. Chem. Biol.* **12**, 402–410 https://doi.org/10.1038/nchembio.2059

60 Kinoshita, M., Suzuki, K.G.N., Matsumori, N., Takada, M., Ano, H., Morigaki, K. et al. (2017) Raft-based sphingomyelin interactions revealed by new fluorescent sphingomyelin analogs. *J. Cell Biol.* **216**, 1183–1204 https://doi.org/10.1083/jcb.201607086

61 Manley, S., Gillette, J.M., Patterson, G.H., Shroff, H., Hess, H.F., Betzig, E. et al. (2008) High-density mapping of single-molecule trajectories with photoactivated localization microscopy. *Nat. Methods* **5**, 155–157 https://doi.org/10.1038/nmeth.1176

62 Kim, D.-H., Zhou, K., Kim, D.-K., Park, S., Noh, J., Kwon, Y. et al. (2015) Analysis of interactions between the epidermal growth factor receptor and soluble ligands on the basis of single-molecule diffusivity in the membrane of living cells. *Angew. Chem. Int. Ed. Engl.* **54**, 7028–7032 https://doi.org/10.1002/anie.201500871

63 Rossier, O., Octeau, V., Sibarita, J.-B., Leduc, C., Tessier, B., Nair, D. et al. (2012) Integrins β1 and β3 exhibit distinct dynamic nanoscale organizations inside focal adhesions. *Nat. Cell Biol* **14**, 1057–1067 https://doi.org/10.1038/ncb2588

64 Lee, Y., Phelps, C., Huang, T., Mostofian, B., Wu, L., Zhang, Y. et al. (2019) High-throughput, single-particle tracking reveals nested membrane domains that dictate KRasG12D diffusion and trafficking. *eLife* **8**, e46393 https://doi.org/10.7554/eLife.46393

65 Goswami, D., Chen, D., Yang, Y., Gudla, P.R., Columbus, J., Worthy, K. et al. (2020) Membrane interactions of the globular domain and the hypervariable region of KRAS4b define its unique diffusion behavior. *eLife* **9**, e47654 https://doi.org/10.7554/eLife.47654

66 Taylor, R.W. and Sandoghdar, V. (2019) Interferometric scattering microscopy: seeing single nanoparticles and molecules via Rayleigh scattering. *Nano Lett.* **19**, 4827–4835 https://doi.org/10.1021/acs.nanolett.9b01822

67 Taylor, R.W., Mahmoodabadi, R.G., Rauschenberger, V., Giessl, A., Schambony, A. and Sandoghdar, V. (2019) Interferometric scattering microscopy reveals microsecond nanoscopic protein motion on a live cell membrane. *Nat. Photon.* **13**, 480–487 https://doi.org/10.1038/s41566-019-0414-6

68 Novotny, L. and van Hulst, N. (2011) Antennas for light. *Nat. Photon.* **5**, 83–90 https://doi.org/10.1038/nphoton.2010.237

69 Levene, M.J., Korlach, J., Turner, S.W., Foquet, M., Craighead, H.G. and Webb, W.W. (2003) Zero-mode waveguides for single-molecule analysis at high concentrations. *Science* **299**, 682 https://doi.org/10.1126/science.1079700

70 Moran-Mirabal, J.M., Torres, A.J., Samiee, K.T., Baird, B.A. and Craighead, H.G. (2007) Cell investigation of nanostructures: zero-mode waveguides for plasma membrane studies with single molecule resolution. *Nanotechnology* **18**, 195101 https://doi.org/10.1088/0957-4484/18/19/195101

71 Kelly, C.V., Baird, B.A. and Craighead, H.G. (2011) An array of planar apertures for near-field fluorescence correlation spectroscopy. *Biophys. J.* **100**, L34–L36 https://doi.org/10.1016/j.bpj.2011.02.034

72 Eid, J., Fehr, A., Gray, J., Luong, K., Lyle, J., Otto, G. et al. (2009) Real-time DNA sequencing from single polymerase molecules. *Science* **323**, 133–138 https://doi.org/10.1126/science.1162986

73 Richards, C.I., Luong, K., Srinivasan, R., Turner, S.W., Dougherty, D.A., Korlach, J. et al. (2012) Live-cell imaging of single receptor composition using zero-mode waveguide nanostructures. *Nano Lett.* **12**, 3690–3694 https://doi.org/10.1021/nl301480h

74 Zhao, Y., Chen, D., Yue, H., Spiering, M.M., Zhao, C., Benkovic, S.J. et al. (2014) Dark-field illumination on zero-mode waveguide/Microfluidic hybrid chip reveals T4 replisomal protein interactions. *Nano Lett.* **14**, 1952–1960 https://doi.org/10.1021/nl404802f

75 Manzo, C., van Zanten, T.S. and Garcia-Parajo, M.F. (2011) Nanoscale fluorescence correlation spectroscopy on intact living cell membranes with NSOM probes. *Biophys. J.* **100**, L8–L10 https://doi.org/10.1016/j.bpj.2010.12.3690

76 de Bakker, B.I., de Lange, F., Cambi, A., Korterik, J.P., van Dijk, E.M.H.P., van Hulst, N.F. et al. (2007) Nanoscale organization of the pathogen receptor DC-SIGN mapped by single-molecule high-resolution fluorescence microscopy. *ChemPhysChem* **8**, 1473–1480 https://doi.org/10.1002/cphc.200700169

77 de Bakker, B.I., Bodnár, A., van Dijk, E.M.H.P., Vámosi, G., Damjanovich, S., Waldmann, T.A. et al. (2008) Nanometer-scale organization of the alpha subunits of the receptors for IL2 and IL15 in human T lymphoma cells. *J. Cell Sci.* **121**, 627–633 https://doi.org/10.1242/jcs.019513

78 van Zanten, T.S., Cambi, A., Koopman, M., Joosten, B., Figdor, C.G. and Garcia-Parajo, M.F. (2009) Hotspots of GPI-anchored proteins and integrin nanoclusters function as nucleation sites for cell adhesion. *Proc. Natl Acad. Sci. U.S.A.* **106**, 18557–18562 https://doi.org/10.1073/pnas.0905217106

79 van Zanten, T.S., Gómez, J., Manzo, C., Cambi, A., Buceta, J., Reigada, R. et al. (2010) Direct mapping of nanoscale compositional connectivity on intact cell membranes. *Proc. Natl Acad. Sci. U.S.A.* **107**, 15437–15442 https://doi.org/10.1073/pnas.1003876107

80 Taminiau, T.H., Stefani, F.D., Segerink, F.B. and van Hulst, N.F. (2008) Optical antennas direct single-molecule emission. *Nature Photonics* **2**, 234–237 https://doi.org/10.1038/nphoton.2008.32

81 van Zanten, T.S., Lopez-Bosque, M.J. and Garcia-Parajo, M.F. (2010) Imaging individual proteins and nanodomains on intact cell membranes with a probe-based optical antenna. *Small* **6**, 270–275 https://doi.org/10.1002/smll.200901204

82 Mivelle, M., van Zanten, T.S., Neumann, L., van Hulst, N.F. and Garcia-Parajo, M.F. (2012) Ultrabright bowtie nanoaperture antenna probes studied by single molecule fluorescence. *Nano Lett.* **12**, 5972–5978 https://doi.org/10.1021/nl303440w

83 Mivelle, M., van Zanten, T.S. and Garcia-Parajo, M.F. (2014) Hybrid photonic antennas for subnanometer multicolor localization and nanoimaging of single molecules. *Nano Lett.* **14**, 4895–4900 https://doi.org/10.1021/nl502393b

84 Sanz-Paz, M., Wenger, J., van Hulst, N.F., Mivelle, M. and Garcia-Parajo, M.F. (2020) Nanoscale control of single molecule förster resonance energy transfer by a scanning photonic nanoantenna. *Nanophotonics* **9**, 4021–4031 https://doi.org/10.1515/nanoph-2020-0221

85 Kinkhabwala, A., Yu, Z., Fan, S., Avlasevich, Y., Müllen, K. and Moerner, W.E. (2009) Large single-molecule fluorescence enhancements produced by a bowtie nanoantenna. *Nat. Photon.* **3**, 654–657 https://doi.org/10.1038/nphoton.2009.187

86 Acuna, G.P., Moller, F.M., Holzmeister, P., Beater, S., Lalkens, B. and Tinnefeld, P. (2012) Fluorescence enhancement at docking sites of DNA-directed self-assembled nanoantennas. *Science* **338**, 506–510 https://doi.org/10.1126/science.1228638

87 Punj, D., Mivelle, M., Moparthi, S.B., van Zanten, T.S., Rigneault, H., van Hulst, N.F. et al. (2013) A plasmonic 'antenna-in-box' platform for enhanced single-molecule analysis at micromolar concentrations. *Nat. Nanotechnol.* **8**, 512–516 https://doi.org/10.1038/nnano.2013.98

88 Puchkova, A., Vietz, C., Pibiri, E., Wünsch, B., Sanz Paz, M., Acuna, G.P. et al. (2015) DNA origami nanoantennas with over 5000-fold fluorescence enhancement and single-molecule detection at 25 μM. *Nano Lett.* **15**, 8354–8359 https://doi.org/10.1021/acs.nanolett.5b04045

89 Wientjes, E., Renger, J., Curto, A.G., Cogdell, R. and van Hulst, N.F. (2014) Nanoantenna enhanced emission of light-harvesting complex 2: the role of resonance, polarization, and radiative and non-radiative rates. *Phys. Chem. Chem. Phys.* **16**, 24739–24746 https://doi.org/10.1039/C4CP03636K

90 Wientjes, E., Renger, J., Cogdell, R. and van Hulst, N.F. (2016) Pushing the photon limit: nanoantennas increase maximal photon stream and total photon number. *J. Phys. Chem. Lett.* **7**, 1604–1609 https://doi.org/10.1021/acs.jpclett.6b00491

91 Khatua, S., Paulo, P.M.R., Yuan, H., Gupta, A., Zijlstra, P. and Orrit, M. (2014) Resonant plasmonic enhancement of single-molecule fluorescence by individual gold nanorods. *ACS Nano* **8**, 4440–4449 https://doi.org/10.1021/nn406434y

92 Khatua, S., Yuan, H. and Orrit, M. (2015) Enhanced-fluorescence correlation spectroscopy at micro-molar dye concentration around a single gold nanorod. *Phys. Chem. Chem. Phys.* **17**, 21127–21132 https://doi.org/10.1039/C4CP03057E

93 Yuan, H., Khatua, S., Zijlstra, P., Yorulmaz, M. and Orrit, M. (2013) Thousand-fold enhancement of single-molecule fluorescence near a single gold nanorod. *Angew. Chem. Int. Ed. Engl.* **52**, 1217–1221 https://doi.org/10.1002/anie.201208125

94 Donehue, J.E., Wertz, E., Talicska, C.N. and Biteen, J.S. (2014) Plasmon-enhanced brightness and photostability from single fluorescent proteins coupled to gold nanorods. *J. Phys. Chem. C* **118**, 15027–15035 https://doi.org/10.1021/jp504186n

95 Wertz, E., Isaacoff, B.P., Flynn, J.D. and Biteen, J.S. (2015) Single-molecule super-resolution microscopy reveals how light couples to a plasmonic nanoantenna on the nanometer scale. *Nano Lett.* **15**, 2662–2670 https://doi.org/10.1021/acs.nanolett.5b00319

96 Lohmüller, T., Iversen, L., Schmidt, M., Rhodes, C., Tu, H.-L., Lin, W.-C. et al. (2012) Single molecule tracking on supported membranes with arrays of optical nanoantennas. *Nano Lett.* **12**, 1717–1721 https://doi.org/10.1021/nl300294b

97 Fabrizio, E.D., Schlücker, S., Wenger, J., Regmi, R., Rigneault, H., Calafiore, G. et al. (2016) Roadmap on biosensing and photonics with advanced nano-optical methods. *J. Optics* **18**, 063003 https://doi.org/10.1088/2040-8978/18/6/063003

98 Liu, J., Jalali, M., Mahshid, S. and Wachsmann-Hogiu, S. (2020) Are plasmonic optical biosensors ready for use in point-of-need applications? *Analyst* **145**, 364–384 https://doi.org/10.1039/C9AN02149C

99 Oh, S.-H. and Altug, H. (2018) Performance metrics and enabling technologies for nanoplasmonic biosensors. *Nat. Commun.* **9**, 5263 https://doi.org/10.1038/s41467-018-06419-3

100 Maccaferri, N., Barbillon, G., Koya, A.N., Lu, G., Acuna, G.P. and Garoli, D. (2021) Recent advances in plasmonic nanocavities for single-molecule spectroscopy. *Nanoscale Adv.* **3**, 633–642 https://doi.org/10.1039/D0NA00715C

101 Perozziello, G., Candeloro, P., De Grazia, A., Esposito, F., Allione, M., Coluccio, M.L. et al. (2016) Microfluidic device for continuous single cells analysis via Raman spectroscopy enhanced by integrated plasmonic nanodimers. *Optics Express* **24**, A180–A190 https://doi.org/10.1364/OE.24.00A180

102 Coluccio, M.L., Gentile, F., Das, G., Nicastri, A., Perri, A.M., Candeloro, P. et al. (2015) Detection of single amino acid mutation in human breast cancer by disordered plasmonic self-similar chain. *Sci. Adv.* **1**, e1500487 https://doi.org/10.1126/sciadv.1500487

103 Belushkin, A., Yesilkoy, F. and Altug, H. (2018) Nanoparticle-enhanced plasmonic biosensor for digital biomarker detection in a microarray. *ACS Nano* **12**, 4453–4461 https://doi.org/10.1021/acsnano.8b00519

104 Portela, A., Calvo-Lozano, O., Estevez, M.-C., Medina Escuela, A. and Lechuga, L.M. (2020) Optical nanogap antennas as plasmonic biosensors for the detection of miRNA biomarkers. *J. Mater. Chem. B* **8**, 4310–4317 https://doi.org/10.1039/D0TB00307G

105 Flauraud, V., van Zanten, T.S., Mivelle, M., Manzo, C., Garcia Parajo, M.F. and Brugger, J. (2015) Large-scale arrays of bowtie nanoaperture antennas for nanoscale dynamics in living cell membranes. *Nano Lett.* **15**, 4176–4182 https://doi.org/10.1021/acs.nanolett.5b01335

106 Punj, D., de Torres, J., Rigneault, H. and Wenger, J. (2013) Gold nanoparticles for enhanced single molecule fluorescence analysis at micromolar concentration. *Optics Express* **21**, 27338 https://doi.org/10.1364/OE.21.027338

107 Dutta Choudhury, S., Ray, K. and Lakowicz, J.R. (2012) Silver nanostructures for fluorescence correlation spectroscopy: reduced volumes and increased signal intensities. *J. Phys. Chem. Lett.* **3**, 2915–2919 https://doi.org/10.1021/jz301229m

108 Punj, D., Regmi, R., Devilez, A., Plauchu, R., Moparthi, S.B., Stout, B. et al. (2015) Self-assembled nanoparticle dimer antennas for plasmonic-enhanced single-molecule fluorescence detection at micromolar concentrations. *ACS Photonics* **2**, 1099–1107 https://doi.org/10.1021/acsphotonics.5b00152

109 Paulo, P.M.R., Botequim, D., Jóskowiak, A., Martins, S., Prazeres, D.M.F., Zijlstra, P. et al. (2018) Enhanced fluorescence of a dye on DNA-assembled gold nanodimers discriminated by lifetime correlation spectroscopy. *J. Phys. Chem. C* **122**, 10971–10980 https://doi.org/10.1021/acs.jpcc.7b12622

110 Pradhan, B., Khatua, S., Gupta, A., Aartsma, T., Canters, G. and Orrit, M. (2016) Gold-nanorod-enhanced fluorescence correlation spectroscopy of fluorophores with high quantum yield in lipid bilayers. *J. Phys. Chem. C* **120**, 25996–26003 https://doi.org/10.1021/acs.jpcc.6b07875

111 Anger, P., Bharadwaj, P. and Novotny, L. (2006) Enhancement and quenching of single-molecule fluorescence. *Phys. Rev. Lett.* **96**, 113002 https://doi.org/10.1103/PhysRevLett.96.113002

112 Flauraud, V., Regmi, R., Winkler, P.M., Alexander, D.T.L., Rigneault, H., van Hulst, N.F. et al. (2017) In-plane plasmonic antenna arrays with surface nanogaps for giant fluorescence enhancement. *Nano Lett.* **17**, 1703–1710 https://doi.org/10.1021/acs.nanolett.6b04978

113 Regmi, R., Winkler, P.M., Flauraud, V., Borgman, K.J.E., Manzo, C., Brugger, J. et al. (2017) Planar optical nanoantennas resolve cholesterol-dependent nanoscale heterogeneities in the plasma membrane of living cells. *Nano Lett.* **17**, 6295–6302 https://doi.org/10.1021/acs.nanolett.7b02973

114 Wawrezinieck, L., Rigneault, H., Marguet, D. and Lenne, P.-F. (2005) Fluorescence correlation spectroscopy diffusion laws to probe the submicron cell membrane organization. *Biophys. J.* **89**, 4029–4042 https://doi.org/10.1529/biophysj.105.067959

115 Winkler, P.M., Campelo, F., Giannotti, M.I. and Garcia-Parajo, M.F. (2021) Impact of glycans on lipid membrane dynamics at the nanoscale unveiled by planar plasmonic nanogap antennas and atomic force spectroscopy. *J. Phys. Chem. Lett.* **12**, 1175–1181 https://doi.org/10.1021/acs.jpclett.0c03439

116 Winkler, P.M., Regmi, R., Flauraud, V., Brugger, J., Rigneault, H., Wenger, J. et al. (2018) Optical antenna-based fluorescence correlation spectroscopy to probe the nanoscale dynamics of biological membranes. *J. Phys. Chem. Lett.* **9**, 110–119 https://doi.org/10.1021/acs.jpclett.7b02818

117 Wenger, J., Conchonaud, F., Dintinger, J., Wawrezinieck, L., Ebbesen, T.W., Rigneault, H. et al. (2007) Diffusion analysis within single nanometric apertures reveals the ultrafine cell membrane organization. *Biophys. J.* **92**, 913–919 https://doi.org/10.1529/biophysj.106.096586

118 Kelly, C.V., Wakefield, D.L., Holowka, D.A., Craighead, H.G. and Baird, B.A. (2014) Near-field fluorescence cross-correlation spectroscopy on planar membranes. *ACS Nano* **8**, 7392–7404 https://doi.org/10.1021/nn502593k

119 Knight, M.W., Liu, L., Wang, Y., Brown, L., Mukherjee, S., King, N.S. et al. (2012) Aluminum plasmonic nanoantennas. *Nano Lett.* **12**, 6000–6004 https://doi.org/10.1021/nl303517v

120 Krasnok, A.E., Miroshnichenko, A.E., Belov, P.A. and Kivshar, Y.S. (2012) All-dielectric optical nanoantennas. *Optics Express* **20**, 20599 https://doi.org/10.1364/OE.20.020599

121 Kuznetsov, A.I., Miroshnichenko, A.E., Brongersma, M.L., Kivshar, Y.S. and Lukyanchuk, B. (2016) Optically resonant dielectric nanostructures. *Science* **354**, 2472 https://doi.org/10.1126/science.aag2472

122 Regmi, R., Berthelot, J., Winkler, P.M., Mivelle, M., Proust, J., Bedu, F. et al. (2016) All-dielectric silicon nanogap antennas to enhance the fluorescence of single molecules. *Nano Lett.* **16**, 5143–5151 https://doi.org/10.1021/acs.nanolett.6b02076

123 Saemisch, L., Liebel, M. and van Hulst, N.F. (2020) Isolating strong nanoantenna–molecule interactions by ensemble-level single-molecule detection. *Nanoscale* **12**, 3723–3730 https://doi.org/10.1039/C9NR08833D

124 Krieger, J.W., Singh, A.P., Bag, N., Garbe, C.S., Saunders, T.E., Langowski, J. et al. (2015) Imaging fluorescence (cross-) correlation spectroscopy in live cells and organisms. *Nat. Protoc.* **10**, 1948–1974 https://doi.org/10.1038/nprot.2015.100

125 van de Linde, S., Heilemann, M. and Sauer, M. (2012) Live-cell super-resolution imaging with synthetic fluorophores. *Annu. Rev. Phys. Chem.* **63**, 519–540 https://doi.org/10.1146/annurev-physchem-032811-112012

126 Prescher, J.A. and Bertozzi, C.R. (2005) Chemistry in living systems. *Nat. Chem. Biol.* **1**, 13–21 https://doi.org/10.1038/nchembio0605-13

127 Mahen, R., Koch, B., Wachsmuth, M., Politi, A.Z., Perez-Gonzalez, A., Mergenthaler, J. et al. (2014) Comparative assessment of fluorescent transgene methods for quantitative imaging in human cells. *MBoC* **25**, 3610–3618 https://doi.org/10.1091/mbc.e14-06-1091

128 König, A.I., Sorkin, R., Alon, A., Nachmias, D., Dhara, K., Brand, G. et al. (2020) Live cell single molecule tracking and localization microscopy of bioorthogonally labeled plasma membrane proteins. *Nanoscale* **12**, 3236–3248 https://doi.org/10.1039/C9NR08594G

129 Neubert, F., Beliu, G., Terpitz, U., Werner, C., Geis, C., Sauer, M. et al. (2018) Bioorthogonal click chemistry enables site-specific fluorescence labeling of functional NMDA receptors for super-resolution imaging. *Angew. Chem. Int. Ed. Engl.* **57**, 16364–16369 https://doi.org/10.1002/anie.201808951

130 Krmpot, A.J., Nikolić, S.N., Oasa, S., Papadopoulos, D.K., Vitali, M., Oura, M. et al. (2019) Functional fluorescence microscopy imaging: quantitative scanning-free confocal fluorescence microscopy for the characterization of fast dynamic processes in live cells. *Anal. Chem.* **91**, 11129–11137 https://doi.org/10.1021/acs.analchem.9b01813