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Quantitative Bio-Imaging Tools to Dissect the Interplay of Membrane and Cytoskeletal Actin Dynamics in Immune Cells

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Cellular function is reliant on the dynamic interplay between the plasma membrane and the actin cytoskeleton. This critical relationship is of particular importance in immune cells, where both the cytoskeleton and the plasma membrane work in concert to organize and potentiate immune signaling events. Despite their importance, there remains a critical gap in understanding how these respective dynamics are coupled, and how this coupling in turn may influence immune cell function from the bottom up. In this review, we highlight recent optical technologies that could provide strategies to investigate the simultaneous dynamics of both the cytoskeleton and membrane as well as their interplay, focusing on current and future applications in immune cells. We provide a guide of the spatio-temporal scale of each technique as well as highlighting novel probes and labels that have the potential to provide insights into membrane and cytoskeletal dynamics. The quantitative biophysical tools presented here provide a new and exciting route to uncover the relationship between plasma membrane and cytoskeletal dynamics that underlies immune cell function.

Keywords: plasma membrane, actin cytoskeleton, fluorescence correlation spectroscopy, fluorescence recovery after photobleaching, immune cells, metal induced energy transfer, volumetric imaging, quantitative imaging

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INTRODUCTION

Life is dynamic. Cellular components are in constant motion bridging various time- and length-scales. This includes the plasma membrane and the cortical actin cytoskeleton, which form a dynamic interface between the cell and its environment, working together to control cellular signaling and morphology as well as to maintain the mechanical integrity of the cell. It is becoming increasingly clear that the dynamics of the cortical actin cytoskeleton and the plasma membrane are intimately linked to immune cell function, playing a critical role in, for instance, the regulation of receptor organization, granule section, and specific cytoskeletal protrusions (1–4). Despite their individual importance, how both the membrane and the cortical actin cytoskeleton dynamics are coupled, and how feedback between the two structures shapes their interplay in immune cells remains unknown. Crucially, to understand the functional significance of this interplay,

measurement techniques are required that allow the dynamics of actin and membrane to be captured simultaneously, enabling their direct correlation in both space and time.

Actin-Membrane Interactions at a Glance

The interactions between the membrane and the actin cortex are numerous and complex and have been the subject of intense research (5, 6) (**Figure 1A**). Underlying the plasma membrane the actin cortex exists as a densely cross-linked meshwork of filamentous actin (F-actin) formed by the polymerization of globular actin (G-actin) monomers undergoing constant turnover on the second time-scale (7). The dynamic architecture of the actin cortex is governed by two primary modes of F-actin polymerization driven by either Arp2/3 or formin nucleation leading to constantly varying actin mesh-sizes from tens of nanometers to microns (8). The dynamic nature of the cortex as well as its mechanical plasticity is largely mediated by a variety of myosin motors that cross-link individual filaments and induce active mechanical stress within the network (8).

Biochemically linking the actin cortex and the plasma membrane are a series of specific protein-protein and protein-lipid interactions (9). One of the most important membrane

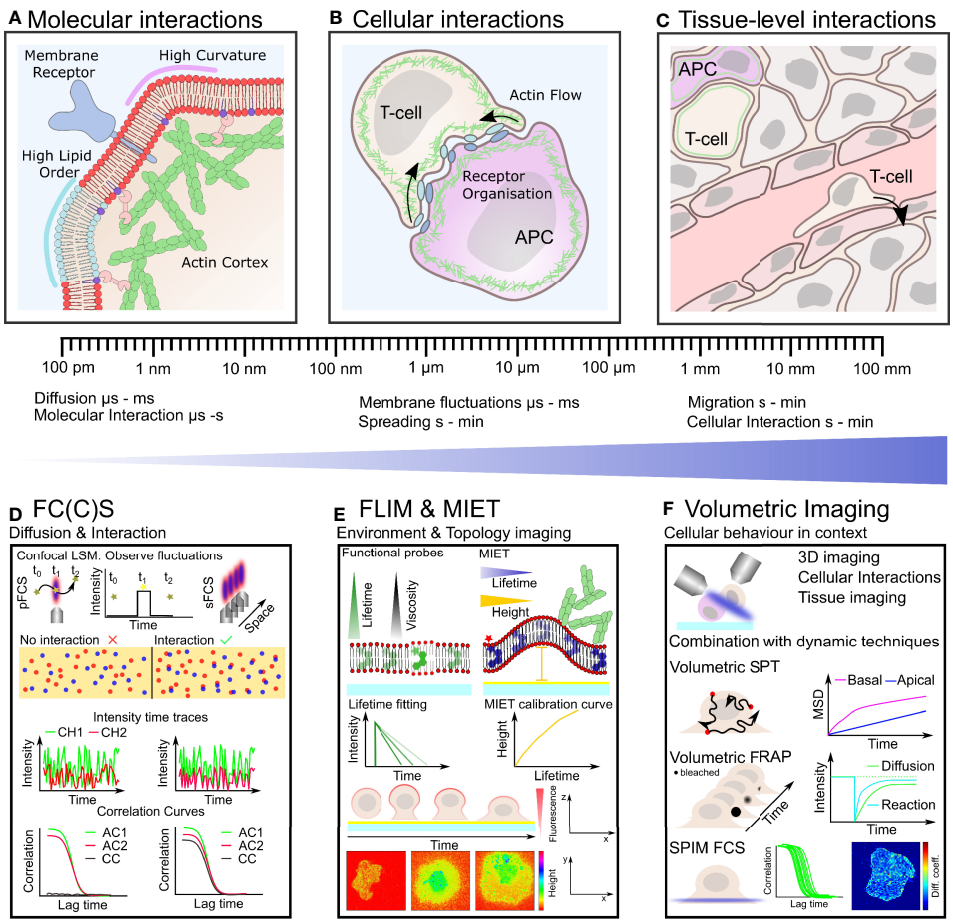

FIGURE 1 | Plasma membrane organization and actin cytoskeletal dynamics are intrinsically linked to immune cell organization and function over many length- and time-scales **(A–C)**. Techniques with the ability to quantify simultaneous dynamics present an exciting route to understand the interplay of cortical actin and plasma membrane in immune cells **(D–F)**. **(A)** On the nano-scale actin filaments are turning over and binding the plasma membrane influencing its organization which in turn impacts on actin organization and the distribution of receptors, lipids and other membrane constituents. **(B)** Defined actin and membrane flows, organization and their integrity are crucial for cell-cell contacts such as during immunological synapse formation on the meso-scale. **(C)** Within tissue, immune cells must navigate through biophysically diverse environments, relying on both a dynamic plasma membrane and actin cytoskeleton to carry out their function. **(D)** Fluorescence correlation spectroscopy (FCS) and FCCS measure fluctuation of fluorescently labeled molecules diffusing through the focus of a confocal microscope. Typical transit times range from µs to hundreds of ms highlighting the large dynamic range and unrivaled temporal resolution of these techniques. Auto-correlation (AC) of the intensity traces (in two spectral channels CH1 and CH2) allows to calculate diffusion coefficients and interactions (cross-correlation, CC). **(E)** Fluorescence lifetime imaging (FLIM) allows to monitor changes in fluorescence lifetime. Acquiring FLIM images typically takes seconds to minutes. Functional probes can change their lifetime in accordance to their environment sensing, for example, viscosity. In metal induced energy transfer (MIET) the lifetime change is correlate with the distance to the surface and allows the recalculation of heights yielding the membrane topology, for example, during cell spreading. **(F)** Volumetric imaging using, for instance, a light-sheet approach allows to image the cellular context in 3D at moderate time resolution (down to seconds). Combination with the dynamics techniques, single particle tracking (SPT), fluorescence recovery after photobleaching (FRAP), and FC(C)S represents a promising route for mapping plasma membrane and actin dynamics in the full physiological setting by correlating their time- and length-scales.

components mediating this interaction is the glycolipid phosphatidylinositol-bisphosphate, PIP2. By binding the ERM (ezrin, radixin, and moesin) proteins, an actin binding family of proteins, PIP2 provides a linkage between cytoskeleton and membrane (**Figure 1A**). In addition to the ERM proteins, WASP and WAVE, key regulators of Arp2/3 driven actin polymerization, are also able to bind PIP2 in the membrane, leading to active polymerization of F-actin at the plasma membrane (6). Notably, the conserved diversity of specific linkages between cortex and membrane is indicative of the importance of the tight coupling of these structures for their function within the cell.

In addition to the specific molecular interactions between the plasma membrane and actin cortex, there are a number of more general biophysical interactions that have been well characterized *in vitro* (10), for example, the local charge of the membrane has been shown to influence actin binding (11). Furthermore, curvature introduced by the polymerization of the actin cytoskeleton during the formation of specific protrusions can influence the diffusion and distribution of membrane proteins (12, 13). Conversely, membrane curvature induced by the physical membrane microenvironment can lead to actin polymerization (14). Local changes in actin polymerization can also induce changes in the rate and diffusion mode of lipid and protein components as well as the membrane tension (15–18). Crucially, membrane composition, dynamics, and organization influence the underlying actin cytoskeleton. Similarly, the dynamics and architecture of the actin cytoskeleton has been shown to influence the plasma membrane (19). Therefore, the complex interplay between membrane and cortical cytoskeleton makes assessing causal links challenging, highlighting the unmet need for techniques that allow the dynamics of both components to be quantified simultaneously in space and time.

Actin-Membrane Interactions in Immune Cells

Many stages of the immune response, for example, antigen recognition, rely on the integration of information by immune cells from their environment, often involving the formation of highly specialized cell-cell contacts, such as the immunological synapse (IS) that forms between T-cells, B-cells, and antigen presenting cells (APCs) (**Figure 1B**). At these contacts, immunological signaling is initiated and propagated *via* the interactions of a wide array of molecules, occurring on and in the proximity of the plasma membrane. Owing to this, the dynamics of both the plasma membrane and the underlying actin cytoskeleton have a profound impact on the organization and dynamics of crucial signaling molecules. The process of immune cell activation spans a range of time- and length-scales starting with nano-scale reorganization and receptor engagement (sub-second), actin polymerization driven retrograde flow (seconds), to micron-scale cellular activation, spreading, and cytokine secretion (up to hours) (20–22).

During T-cell activation and IS formation, one of the key steps in the adaptive immune response, there has been increasing interest in the role of specific cytoskeletal protrusions in the initiation and orchestration of early T-cell signaling at the plasma membrane (23–25). Microvilli at the T-cell surface have been shown to provide an efficient means of environment scanning, while association of ERM proteins to the plasma membrane interface has been shown to lead to the accumulation of signaling molecules at microvilli (23, 24, 26). Following T-cell receptor (TCR) triggering, the T-cell undergoes a dramatic morphological change driven by the rapid polymerization of F-actin. This results in the formation of an increased contact area between the two cells, which is characterized by the retrograde flow of actin filaments within a lamellipodial structure at its periphery as well as a ramified actin network at its center (27). Crucially, TCRs are trafficked in coordination with the F-actin flow toward the center of the IS and the continued flow of actin has been shown to be necessary for continued activation, sustaining PLCγ1 signaling (28–30). Notably, once the IS has formed, recent evidence suggests that tension generated by specific dynamic actin structures influence the symmetry and lifetime of the IS (31).

In contrast to its polymerization, the depletion of actin has also been shown to play a role in immune cell function. Targeted killing by cytotoxic T lymphocytes requires the precise spatio-temporal control of actin depletion with recent studies pointing to a complex and intricate mechanism, whereby the density of the cortical actin underlying the membrane is tuned by the interaction of the kinase PIP5K and the loss of the charged PIP2 lipid (32, 33).

Like the cortical actin cytoskeleton the plasma membrane is a dynamic structure, constantly in motion and continuously reorganizing (34, 35). The presence of defined domains or rafts (tens of nanometer in diameter) has been evoked to explain a number of phenomena, including lateral heterogeneity of the plasma membrane and, for example, the non-random distribution of membrane proteins on the cell surface (35–38). Along these lines, the reorganization of immune receptors by specific incorporation into such structures has been described (39–41). This is based on a biophysical property of the membrane and the proteins themselves: proteins can exhibit a preference for different lipid environments preferring, for instance, a densely packed lipid environment (liquid ordered phase, highly viscous) enriched in saturated lipids and cholesterol (42, 43). In contrast, other proteins can prefer the liquid disordered membrane environment, rather associating with loosely packed, unsaturated lipids resulting in a low viscosity environment. This could act as means of increasing interaction likelihood and forming signaling platforms (44, 45). The degree of order and the viscosity of the membrane are primarily tuned by the cholesterol content and in addition likely by its specific interactions with proteins and lipids (46–48). Intriguingly, the attachment of actin filaments to the membrane has been seen to influence membrane organization, resulting in the formation of ordered domains (49, 50), providing a potential mechanism for actin to indirectly influence membrane protein organization.

As is evident, studies of the dynamics of the cytoskeleton and membrane have led to important insights into the function of immune cells (51–53). Despite this, there are only a limited number of studies that have attempted to address the correlated biophysical and biochemical dynamic mechanisms whereby membrane and actin work together in immune cells. Recent advances in correlative imaging such as the combination of super-resolution microscopy and electron microscopy have allowed for detailed insights into

structural links between plasma membrane and cortical actin organization (54–56). However, these approaches only allow snapshots of a constantly evolving structure and thus do not allow the dynamic interplay to be followed live, and therefore make assessing causality challenging. Furthermore, little is known about how these interactions influence the behavior of cells in more complex tissue environments or in their full physiological setting, with our knowledge often restricted to *in vitro* single cell studies (**Figure 1C**). This has primarily been due to a lack of accessible technologies with sufficient temporal (< ms binding and transport events) and spatial resolution (single proteins on the order of nanometres can effect changes on cellular level beyond tenth of micrometers) to assess the correlated dynamics of the plasma membrane and the actin cytoskeleton without perturbing the system. In addition to this, such lack of technology has been confounded by a lack of membrane and actin probes that can operate at physiological conditions and offer reliable performance within the cellular environment.

Here, we review recent advances in both dynamic measurement techniques and actin/membrane probes that have not yet been widely applied to study immune cells. In our view, these methods present a significant opportunity to address the complex interplay between these two systems crucial to the immune response by simultaneous quantification of both membrane and actin dynamics.

FROM FLUORESCENCE IMAGING TO QUANTIFICATION OF DYNAMICS

Live-cell fluorescence imaging is the method of choice to understand the behavior of dynamic biological processes owing to the specificity of labeling structures of interest and the minimal invasiveness of the approach. Observing events crucial to the immune response occurring in live cells has long been performed by employing fluorescence microscopy with confocal and total internal reflection fluorescence (TIRF) time-lapse imaging due to their optical sectioning capabilities yielding key insights into the dynamic nature of these processes (57, 58). This approach has revealed, for example, the formation and trafficking of T-cell receptor clusters (21, 59), which is crucial for initiation and continuation of signaling and has been shown to be strongly regulated by the dynamic interplay of the membrane and cortical actin cytoskeleton flows (60, 61). Despite their success, imaging alone restricts the level of quantitative information that can be extracted from the biological system of interest, for instance, due to the limited time resolution of time-lapse imaging acquisitions (tens of ms to s).

An alternative quantitative route to assessing transient processes such as cellular reorganization driven by molecular diffusion, binding-kinetics, or flow has been the use of dynamic techniques such as single particle tracking (SPT), fluorescence recovery after photobleaching (FRAP), or fluorescence fluctuation spectroscopy (FFS) approaches. For a detailed technical description, we refer the reader to (62–64). In immunology, these traditional techniques and their advancements have, for instance, been used to investigate the diffusion properties of key signaling molecules such as BCR, CD1d, TCR, CD45, or Lck in live cells (65–74).

Fluorescence Fluctuation Based Approaches to Assess Simultaneous Dynamics

The spatial heterogeneity across microns along with fast molecular interactions within the cell membrane represent a challenge to all dynamic techniques which rely on maintaining single molecule sensitivity in the crowded cellular environment. Similar to fluorescence time-lapse imaging FRAP and SPT are conventionally limited to resolving processes in the ms time regime. Using fluctuation based techniques such as fluorescence correlation spectroscopy (FCS) offers unmatched temporal resolution (down to ns) to cover the range from very fast molecular binding dynamics (μs) to motion of large protein complexes in the membrane (hundreds of ms). Unfortunately, the spatial resolution remains diffraction limited (~200 nm). Thus, inherently one will average many molecular interactions missing precise details on dynamics and potential sub 100 nm spatial heterogeneity which could be functionally important, for instance, during receptor-ligand engagement. The combination of stimulated emission depletion (STED) super-resolution microscopy with FCS has proven itself as a valuable remedy and a tool to assess nano-scale diffusion directly at the relevant spatio-temporal scales (75, 76). It has revealed a vast heterogeneity in diffusion behaviors of membrane constituents caused by interactions with lipid domains, transmembrane proteins, or the cortical actin cytoskeleton (16, 17, 77). While STED-FCS offers very high spatial resolution in living specimens (<50 nm), it is limited by comparatively high light exposure, requires dedicated equipment (depletion beam), and necessitates special dyes (75).

By sampling not only a single point, but rather a linear or circular region, scanning fluorescence correlation spectroscopy (sFCS) studies offer not only increased statistical power over conventional FCS but also limited phototoxicity (78, 79), and allow spatial heterogeneities in molecular diffusion dynamics to be accounted for (**Figure 1D**). The increased statistical power of sFCS can be exploited to decipher the diffusion mode of the molecule of interest (determining if a molecule undergoes free Brownian or hindered diffusion (80) due to nano-scale interactions). On the one hand, changes in diffusion behavior can initiate signaling pathways and on the other hand can be indicative of a cellular state such as activation (81, 82). Similar to STED-FCS, the statistical analysis of sFCS data can yield details of dynamic molecular organization but notably does not rely on any special equipment or dyes and can be performed on any turn-key confocal laser scanning microscope (79, 80). Critically, two-color scanning fluorescence cross-correlation spectroscopy (sFCCS) data can be used to characterize the dynamics of two species of interest, exploring their interplay in detail (83–86) (**Figure 1D**). Super-resolved (STED) cross-correlation studies have not yet been achieved, but in combination with beam scanning bare potential to uncover short-lived interactions (87, 88). Together with harnessing the statistical power of scanning approaches, we anticipate that the advances in fast photon-counting acquisitions,

high-count rate FCS and thus fast photon filtering may pave the way to the realization of such techniques (89, 90). Cross-correlation studies offer a unique opportunity to quantify cytoskeletal and membrane dynamics simultaneously, for example, probing the dynamic interactions between signaling molecules at the plasma membrane and the flow of the actin cytoskeleton during synapse formation, allowing cross-correlation on short (μs to ms) time-scales. Such correlated, simultaneous acquisitions represent a promising way to dissect causation and may decipher when the actin cytoskeleton is driving the membrane organization and vice versa.

Imaging larger regions allows for the mapping of diffusion across space and delivers increased spatial information at the expense of temporal resolution (> ms). Both TIRF and single-plane illumination (SPIM) schemes have been combined with camera based FCS acquisitions yielding similar and even larger statistics compared to sFCS (91–93). Crucially, these techniques provide subcellular or cellular imaging, contextualizing the dynamic measurements and allowing routine correlation with specific compartments of the cell. Expanding such approaches using image mean squared displacement (iMSD) analysis can even yield insights into the diffusion modes with a statistical power similar to STED-FCS and sFCS (94, 95). For camera based acquisitions the frame time (of about 1 ms minimum) represents the most common bottleneck for resolving fast diffusion (96). Given recent advances in camera technology this will likely not pose a limitation for much longer. For example, interferometric scattering (iSCAT) microscopy, which relies on collecting scattered light from the sample rather than fluorescence emission, allows frame rates of multiple kilo Hertz covering most of the range of dynamic processes in biology (97–99).

Exploiting Fluorescence Lifetime to Measure Dynamics and Topology

The time a fluorophore spends in the excited state is termed fluorescence lifetime and can be used as an additional means for introducing contrast in fluorescence microscopy with the fluorescence lifetime strongly depending on the environmental conditions and fluorophore properties (62). The unrivaled temporal resolution of fluorescence fluctuation approaches offer a promising route to decipher lateral membrane organization. Yet, biology operates in all three spatial dimensions, for example, actin polymerization causing plasma membrane deformations, and the aforementioned methods are largely blind to changes in axial organization. In the following, we discuss a possible remedy exploiting fluorescence lifetime modulation.

The recent advances in commercially available fluorescence lifetime imaging (FLIM) platforms enable fast acquisitions (few seconds per frame) and easy access to this microscopy modality (100). Typically, fluorescence lifetime information is used as an intrinsic method to generate contrast in unlabeled samples (using auto-fluorescence) or in conjunction with Foerster resonance energy transfer (FRET), where the lifetime shortening of the fluorescence donor is used to calculate the distance between two fluorochromes (donor and acceptor) revealing molecular interactions or conformational changes (101) (**Figure 1E**). A versatile variation of FRET makes use of the lifetime and fluorescence quenching abilities of thin metal films on the glass coverslip. In metal induced energy transfer (MIET), the lifetime can be used to calculate the height (distance from the quenching surface) of a fluorophore with nanometer precision across a range of 0-150 nm (102) (**Figure 1E**). The dynamic range and localisation precision can be tuned by the coating material (most commonly gold and more recently graphene) (103–105). MIET displays a great opportunity to explore membrane topology and curvature (106), for example, in common T cell surface interaction studies using plasma membrane markers, as has been applied to study the epithelial-to-mesenchymal transition of epithelial cells (107). This becomes even more powerful when combined with two-color labeling (108, 109), allowing the simultaneous spatio-temporal quantification of the actin cortex and plasma membrane topology, which may elucidate microvilli structures and allow axial mapping of segregation and IS organization (109). Specifically, it could be used to differentiate actual protein clusters from axially stacked molecules (as in microvilli). MIET can be combined with FCS (or rather its cousin fluorescence lifetime correlation spectroscopy) allowing for height dependant dynamic measurements, giving the opportunity to separate receptor dynamics proximal and distal from the surface within one measurement in a few seconds, allowing key insights into the mobility of receptors in the vicinity of specific actin structures (110). As a further extension, the combination of fluorescence lifetime imaging with functional probes paves the way for some exciting applications, whereby membrane topology can be correlated with other readouts, such as membrane tension, curvature, or order (see below).

Fast Volumetric Imaging and Its Combination With Dynamic Techniques

The aforementioned approaches give highly detailed insights on fast time- and short length-scales. Nevertheless, the actin cortex plasma membrane interplay also affects larger-scale cellular dynamics such as pushing/pulling of the membrane, cell migration and sampling of the immediate surroundings (see introduction) (**Figure 1C**). The investigation of these processes necessitate tools that are able to operate in in all three dimension (3D), capturing the complex geometries and topologies of cellular and multicellular samples. In biology, 3D (volumetric) imaging has typically been achieved using axial scanning confocal microscopy, and more recently using super-resolution techniques such as 3D-SIM. Unfortunately, such techniques are often restricted to relatively long (several seconds to minutes) scan times, limiting their application to slowly evolving biological systems. To investigate transient processes in a more physiological setting (compared to a planar coverslip), great advances in volumetric and *in vivo* imaging have been made, primarily based on the use of light sheet technologies, allowing for rapid 3D acquisition (111–116) (**Figure 1F**). These volumetric imaging approaches present an opportunity to overcome a long-standing issue for the investigation of plasma membrane and actin cortex interactions in lymphocytes such as T-cells. Typically, due to restrictions imposed by conventional imaging methods, specific activation is achieved by replacing the antigen presenting cell by a coverslip coated with an activing molecule or a supported lipid

bilayer presenting target molecules (117, 118). This approach has led to a great number of important insights into immune cell biology, yet it omits a large proportion of the biological complexity and three-dimensional geometry present within the physiological interactions between immune cells and target cells. Consequently, the opportunity to investigating plasma membrane and actin cortex interactions in physiological geometry using volumetric imaging is likely to yield great insights (33, 119, 120).

Crucially, instead of acquiring time-lapse imaging alone, combing the aforementioned dynamic techniques such as FCS, FRAP, or SPT with light-sheet imaging has the great advantage of providing spatial context for the observations (121). Notably, in contrast to FCS, the FRAP method can not only extract the dynamics of one species of interest, but can also quantify reaction processes, such as the binding of actin monomers within the actin cortex (122). The combination of FRAP with volumetric imaging therefore represents an exciting opportunity to correlate diffusive processes, for example, at the plasma membrane with the reaction driven turnover of the actin cortex beneath. Such technologies will likely be key in providing insights into the correlated dynamics of immune cell membrane and actin cytoskeleton within physiologically relevant environments (123).

All these techniques and ideas, of course, rely on appropriate and non-perturbing labeling strategies. Excitingly, a large variety of actin and membrane labels are now available.

Labels and Probes for Quantification of Plasma Membrane Dynamics

A variety of approaches can be chosen to label the plasma membrane. Broadly speaking, labels can be divided in specific labels, binding to or mimicking a certain lipid, and non-specific labels, displaying usually hydrophobic compounds, which insert into the membrane. The former can be used to study a specific lipid or pathway, the latter as a general membrane label.

Non-specific labels such as DiO or DiI have been around for decades and have even been used for *in vivo* cell tracking (124, 125). They conveniently incorporate quickly into the membrane by incubation alone which works well with model membranes but can require optimisation for live-cell membrane staining (126). Homogeneous membrane labeling can also be achieved using various other commercial compounds such as the CellMask™ dyes (Life Technologies). More recently, the MemBright dyes were developed allowing for higher photo-stability, lower working concentration and super-resolution microscopy applications (127). Alternatively, specific labels can be used to mimic the structure of a lipid or membrane constituent. This can be a lipid modified with an organic dye, a protein domain specifically binding to a lipid or even an antibody (126, 128). Labeled lipid analogs have enabled insightful studies of the dynamic nano-scale organization of the membrane (76, 77, 87). Nevertheless, due to the comparable size of dye and lipid it cannot be excluded that the analog does not exactly represent the native lipid. An important constituent of the plasma membrane with an abundance of about 30%–40% is cholesterol (35, 46). A variety of probes and cholesterol binding proteins are available (47, 129), however how cholesterol is distributed laterally and axially in the plasma membrane remains under heated debate.

It should be noted that fluorescent WGA conjugates are commonly used to stain the plasma membrane. This protein displays a lectin and rather binds the membrane adjacent glycocalyx thus spatial variations can occur within one cell and definitely when comparing different cell types (127). Some proteins have specific domains to interact with lipids. Such proteins can be tagged with a fluorescent protein and in this way be engineered to become a lipid reporter. These are convenient probes as the cells do not require any further labeling, but overexpression of such reporters may sequester the native lipid species, infer with endogenous signaling and membrane binding and lipid species specificity is often modulated by multiple components. Lipid binding domains include C1-domain (DAG), C2-domain (phospholipids), FYVE- and PX-domain (PI3P), PH-domain (phosphoinositide polyphosphates), and Annexin V (PS) (128, 130, 131).

A variety of compounds exists that not only label the membrane but also report on its properties (environment-sensitive dyes), known as functional probes. Polarity-sensitive dyes, for example, change their fluorescence properties depending on the local order (molecular packing, accessibility of the hydrophobic core to water from the exterior medium) of the surrounding membrane (132, 133). This results in quantifiable changes in fluorescence spectra or lifetimes and can be combined with super-resolution microscopy (134, 135). Other probes sense different membrane properties such as tension or viscosity (136, 137). Work with probes that change their spectrum upon changes in the local environment is experimentally convenient as it only requires acquisition in two optimized spectral channels (even though spectral detection is preferred) (138). Unfortunately, these dyes typically have a very broad emission spectrum that makes it difficult to simultaneously quantitatively image another structure such as actin in addition. Therefore, we anticipate again that lifetime-based probes and acquisitions may show better sensitivity (139, 140).

Approaches for Quantifying F- and G-Actin Dynamics

Visualizing the dynamics of the actin cytoskeleton in living cells remains challenging, largely owing to the rapid turn-over of the molecular components of the cytoskeleton. Two main strategies exists for the visualization of actin within living cells: either genetic approaches modifying G-actin monomers directly with a fluorescent protein or a self-labeling tag (SNAP/Halo), or by using a variety of indirect filamentous F-actin binding labels, for example, the short peptides Lifeact or F-tractin, which are additionally modified with a fluorescent protein (141–143). As with lipid labeling, care must be taken to use the appropriate strategy for the desired dynamic output, for instance, FRAP or FCS studies assessing actin diffusion must utilize a direct G-actin approach to ensure that the measured diffusion is that of G-actin and not the indirect actin probe e.g. Lifeact-filament binding. A recent versatile approach is the visualization of membrane proximal F-actin by a membrane-bound F-tractin reporter (MPAct) as this directly reports on plasma membrane cortex interactions (144).

This enables to investigate actin cortex remodeling in proximity of the membrane, which could yield novel insights into actin-rich protrusions and their dynamic reorganization during T-cell activation. Labeling F-actin in live cells can also be performed with a small organic molecule, SiR-actin (143). This is derived from a toxin and has the advantage that it can simply be added to the cell of interest, is cell permeable and can readily be used with super-resolution STED microscopy (145). Because of its mode of binding to actin filaments, it may perturb native actin dynamics. A rather recent approach displays the use of anti-actin nanobodies (Chromotek) (146–148) or direct delivery of mRNA encoding for labeled Lifeact with certain advantages for primary cells (IBIDI). In immunology both Lifeact and F-tractin have been widely applied to visualize F-actin dynamics (141, 149). More recently, Lifeact has also been implicated in changing endogenous actin dynamics (150–152). As with any secondary labeling, careful optimisation and controls are necessary. When investigating F-actin a comparison with a (fixed) phalloidin stain can serve as a control.

Common to all labeling strategies applied to fluorescence microscopy in living cells should be the use of probes that do not disturb the native behavior of the tagged molecule and do not influence the biological system. As mentioned above, lipids and the attached fluorescent dyes are of a comparable size, and thus measures should be taken to ensure that the lipid behaves as its native counterpart. Often a variety of chemical structures need to be screened in order to optimize labeling of a target molecule (i.e. signaling function, cellular localisation, molecular interactions and so forth need to be preserved and checked) (77, 129). Similar issues arise with the use of fluorescent proteins which can sterically hinder their target protein or artificially cause oligomerisation (153). Therefore, it is advisable to use flexible linkers and trial multiple labeling strategies, for example, C- and N-terminal tagging (154, 155). Overall, we would like to emphasize that controls ensuring the preserved function are of the utmost importance.

Perturbation Studies Using Biochemical- and Photo-Manipulation

Specific labeling of molecules allows the membrane or the cytoskeleton to be studied in different physiological settings. A common additional step of the analysis is to perform perturbations, disturbing the steady-state of the actin assembly or membrane structure or even the actin-membrane interplay. By systematically perturbing different components of the system independently, the impact of each on the signaling and function within the living cell can be inferred and key-components, for example, as drug targets be identified.

Perturbations at the plasma membrane are commonly performed by altering the lipid composition. For transient changes, lipid species can simply be fed but recent evidence shows that the cells quickly counteract this to preserve the biophysical properties of the membrane (156). More commonly, certain membrane constituents are depleted. For instance, cholesterol which is a major component of the plasma membrane, playing an important role in signaling, and is proposed to be the major organizer of nanodomains aka rafts and has a profound effect on the overall biophysical properties such as viscosity and rigidity of the plasma membrane is a common target (46, 157). Treatments with cholesterol oxidase or methyl-β-cyclodextrin are routinely used to deplete cholesterol directly at the plasma membrane (158) for studying fast time scales such as seconds to minutes. Alternatively, drugs such as statins which interfere with cholesterol synthesis in the cell enable investigations of longer time scales up to hours and days (35). In that way, changes in cellular function, membrane organization or signaling upon variations in cholesterol content can be probed (77, 159). Analogously the role of sphingomyelin can be studied by treating the cell with sphingomyelinase or drugs such as fumonisin B1 or myriocin, respectively (35, 47, 77, 160).

Similarly, the actin cytoskeleton and its turnover can be targeted with various drugs or genetically modified (161, 162). For instance, Latruncullin B along with Cytochlasins or Phalloidin can be used to rapidly block F-actin polymerization (163), specifically inhibiting the addition of new actin monomers to the barbed end of F-actin (27, 28, 163). In contrast, Jasplakinolide can be used to stabilize actin filaments and promote polymerization (2, 164). Other drugs can be used to tune the cortex structure by influencing the nucleation of F-actin. For example, CK666 can inhibit actin branching by binding to Arp2/3 which can in turn be used to study the influence of the cortex organization and ultra-structure on the nano-scale diffusion behavior within the membrane (16, 165, 166). Furthermore, the formin inhibitor SMIFH2 can be used to remodel actin filaments and cortex structure (167, 168). In addition, a number of drugs can be used to target myosin molecular motors that drive stress generation within cortex. Specifically, both blebbistatin (169), an inhibitor of myosin II ATPase activity, and the rho kinase inhibitor Y27632 (170), are commonly used to perturb the ability of myosin II to actively generate stress within the cortex.

While these studies provided profound insights, for example, into actin reorganization during T-cell activation (27, 168), such perturbations using small molecules and enzymes affect the cell as a whole and can have unwanted side effects: For example, SMIFH2 has recently been indicated to inhibit myosins in addition to formins (171). Because of this, care needs to be taken when interpreting their effect on the structure or process of interest. A remedy to looking at global changes may come with the introduction of more photo-caged compounds or photo-activatable proteins, offering the potential for spatio-temporal control of perturbations (172–174). In addition, model systems such as cell-derived vesicles offer more control but do not allow to measure a living system (17, 35, 175).

DISCUSSION AND FUTURE PERSPECTIVES

The communication of immune cells with their environment, other immune cells and target cells involves a diversity of complex receptor-ligand interactions. These interactions all take place within the context of the plasma membrane and the underlying actin cortex. Consequently, their dynamics are intimately linked to the biophysical properties of both the membrane and the actin cytoskeleton and are constantly influencing one another. In this review, we have sought to highlight tools and technologies that present exciting opportunities to uncover the correlated dynamics

of the plasma membrane and the cortical actin cytoskeleton at the immune cell interface for the first time, harnessing the power of simultaneous acquisitions. In particular, the presented methodologies provide a route to pick apart the key determinants of actin-membrane dynamics, unraveling the causal mechanistic relationships between the two systems. Crucially, these technologies operate across a wide range of length- and time-scales, allowing the investigation of nanoscale interactions on the sub-millisecond time scale up to large scale whole-cell measurements using volumetric imaging. Armed with these new technologies, immunologists can address key questions regarding the interactions of molecules on or in the proximity of the plasma membrane. The ubiquity of membrane interactions in immune cell functions means these techniques have the potential to provide insight into a wide range of immunological cell types, in both the adaptive and innate immune response.

As detailed, these new technologies together with novel functional probes allow the assessment of important biophysical parameters such as lipid order, charge, viscosity, and membrane tension. Many of these parameters have been implicated in immune cell function, thus their systematic probing using these newly available tools is timely (176–178). Coupled with established immunological methods, such as fluorescence-activated cell sorting (FACS), these techniques provide a powerful route to better understand the function of a wide range of immune cells.

To maximize the gains from the presented techniques, they must be applied at the appropriate time- and length-scales. FCS, for instance, operates at the μs to ms time scales and sub-micron spatial-scales, and FRAP rather on ms to s and on micron scales. MIET offers high sensitivity in the axial direction, but remains diffraction limited laterally, and temporal resolution is limited by the sample signal and lifetime acquisition (~s). Volumetric imaging can be extremely rapid, but in most cases cannot surpass the diffraction limit in spatial resolution. 3D-SIM is promising in the regard of isotropic sub-diffraction resolution but sacrifices temporal resolution (179, 180). Therefore, care needs to be taken to answer the right questions with the right tools. In addition to this, as discussed, probes and labels should be chosen such that they maximize the potential of the applied technique, for example, in FCS, dyes with a high molecular brightness and high photo-stability are desirable, whereas dyes utilized for FRAP should allow for efficient photobleaching. For MIET, fluorescent dyes showing a single exponential lifetime decay curve are desirable to allow for more straightforward reconstruction of the topological features from the quenched lifetimes. In addition, the labeling density should be considered: SPT, for example, can only be applied in the case that single molecules (single emitters) can be tracked, whereas FRAP and FCS can operate over a much wider range of molecular densities. Lastly, as for any multi-color microscopy experiments, the emission spectra and the possible overlap of the utilized dyes should be taken into account. Especially for dynamic techniques spectral bleed-through can result in the measurement of false positive interactions.

While these techniques present exciting opportunities for single cells and subcellular context, future work should focus on extending the capabilities of these methods to operate in more complex, more relevant multi-cellular environments including tissues and living organisms. Indeed, work in this direction is well underway with the introduction of rapid volumetric imaging systems like those presented here. We believe that great potential lies in the combination and integration of large scale volumetric imaging with technologies such as FC(C)S, FRAP and SPT, providing quantification of key biophysical parameters throughout the functionally diverse life cycles of immune cells.

AUTHOR CONTRIBUTIONS

FS, HC-Y, and MF wrote the manuscript. All authors contributed to the article and approved the submitted version.

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