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Review Article

Understanding immune signaling using advanced imaging techniques

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Advanced imaging is key for visualizing the spatiotemporal regulation of immune signaling which is a complex process involving multiple players tightly regulated in space and time. Imaging techniques vary in their spatial resolution, spanning from nanometers to micrometers, and in their temporal resolution, ranging from microseconds to hours. In this review, we summarize state-of-the-art imaging methodologies and provide recent examples on how they helped to unravel the mysteries of immune signaling. Finally, we discuss the limitations of current technologies and share our insights on how to overcome these limitations to visualize immune signaling with unprecedented fidelity.

Introduction

Immune signaling processes occur over broad spatial and temporal scales. The spatial reorganization of immune signaling ranges from large micron-sized patterns, termed supramolecular activation clusters (SMACs) to signaling micro-clusters and nanoclusters below the resolution limit of conventional light microscopy [1,2]. The temporal events can span from microsecond exploratory interactions to stable cell–cell contacts lasting over minutes. One single imaging technique cannot cover all spatial and temporal scales simultaneously and it is necessary to compromise in certain aspects to gain in others (Figure 1). Therefore, different modes of cellular imaging are used to enable visualization of specific molecules and to elucidate their interplay, structure and the resulting biological signaling pathways without significant perturbation of the fragile machinery of the immune response. In this review, we summarize recent technologies that provide insight into the molecular details of immune cell signaling. We cover dynamic imaging for long-term visualization, super-resolution microscopy beyond the diffraction limit and biophysical imaging for measuring collective cellular properties. By showcasing recent examples of how these techniques have been used to unravel the molecular details of immune signaling, we aim to provide a general understanding of this unique toolbox for immunologist (Figure 2).

Dynamic imaging of immune signaling

The triggering of the T-cell receptor (TCR) occurs within a few seconds of cell–cell contact [1], which involves reorganization of membrane proteins over 20–100 nm [3] and the fast motion of finger-like microvilli structures [4,5] that continuously change in shape [6]. These conditions all together present highly technical imaging challenges. As every microscopy technique has their own advantages and limitations (Figure 1), the dynamic imaging of immune signaling processes has been approached with a variety of microscopy techniques.
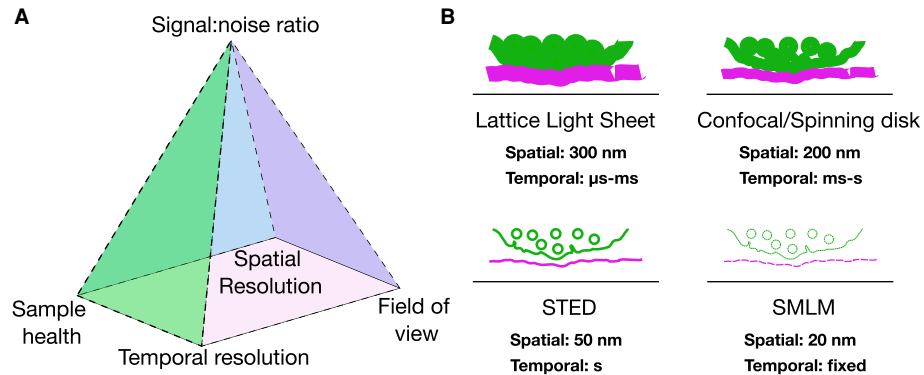
Figure 1. Demands for different microscopy techniques.
(**A**) Pyramid of frustration in microscopy. One single imaging technique cannot cover all axes of organismal, cellular and molecular imaging. Compromises from certain aspects are common to gain in others. (**B**) Imaging modalities discussed in this review and their comparison in spatiotemporal resolution.

Imaging the interface

The interface between a glass coverslip and immune cells presents an attractive platform for imaging signaling processes occurring at the plasma membrane. Total internal reflection fluorescence microscopy (TIRFM) can be applied to create an evanescent wave that penetrates 100–200 nm into the cell [7], enabling high-contrast imaging of the glass-cell interface. This has been used to visualize formation of SMACs [8], nanoscale reorganization of T-cell membrane proteins [3] and formation of microclusters in B cells [9] and T cells [8]. Naturally, the high contrast of TIRFM lends itself to single-molecule imaging of membrane proteins [10] (covered in the subsequent super-resolution section). While TIRFM achieves high axial resolution, it is limited to 2D with diffraction-limited resolution. By controlling the incident angle of the laser beam reflected off the coverslip glass, the penetration depth of the evanescent beam can be varied to gain 3D information. Such variable-angle (VA)-TIRFM [11] has been used to study the distribution and the size of membrane proteins and membrane topography [3,11]. Like with TIRFM, the topography of the glass-membrane interface can also be measured by taking advantage of the refractive index change between the cell membrane and the cell media. Interference reflection microscopy (IRM) is a label-free technique that has been applied to study adhesion of immune cells to model antigen-presenting cell surfaces [3,13]. By coating the surface with a reflective metal and varying the incident angle to enable scanning angle interference microscopy (SAIM), it is also possible to perform axial measurements with nm precision [14]. This precision is key for discerning topographical variations in membrane protein distributions and it has been applied to reveal that TCR microclusters are enriched closer to the activating interface as opposed to bulk TCR [6].

These methods all attempt to model a cell–cell interface, typically by coating the coverslip with a supported lipid bilayer (SLB) that incorporates membrane proteins. However, this presents challenges as the physiological relevance of SLBs can be questioned due to unknown role of the glycocalyx [15], unexpected cell activation [13,16] and the effect of the hard glass on mechanosensitive cells [17]. To minimize artifacts, immune signaling should ideally be studied in 3D and preferably at the intercellular interface.

Dynamic 3D imaging

Fluorescence images of an immunological synapse (IS) were first acquired using confocal microscopy in the late 90s [18,19]. Commercial spinning disk confocal microscopes routinely achieve diffraction-limited resolution (lateral: 200 nm, axial: 500 nm) with video rate 2D imaging (20 Hz) or slightly slower 3D imaging (∼1 Hz). Most immune signaling studies have relied on this imaging technique with examples including demonstration of finger-like structures breaking through the cell membrane glycocalyx [20], the release of cell-killing attack particles [21] or calcium influx [13]. The main disadvantages of confocal microscopy are the limited speed and the propensity for photobleaching (Table 1). Multi-point detector confocal techniques such as Airyscan super-resolution imaging [22] have been able to effectively double the spatial resolution, which has enabled dynamic 2D imaging to resolve B cell synapse formation [23]. However, the ∼10-fold reduction in speed has been prohibitive for dynamic 3D imaging of larger structures, such as the cell–cell interface.
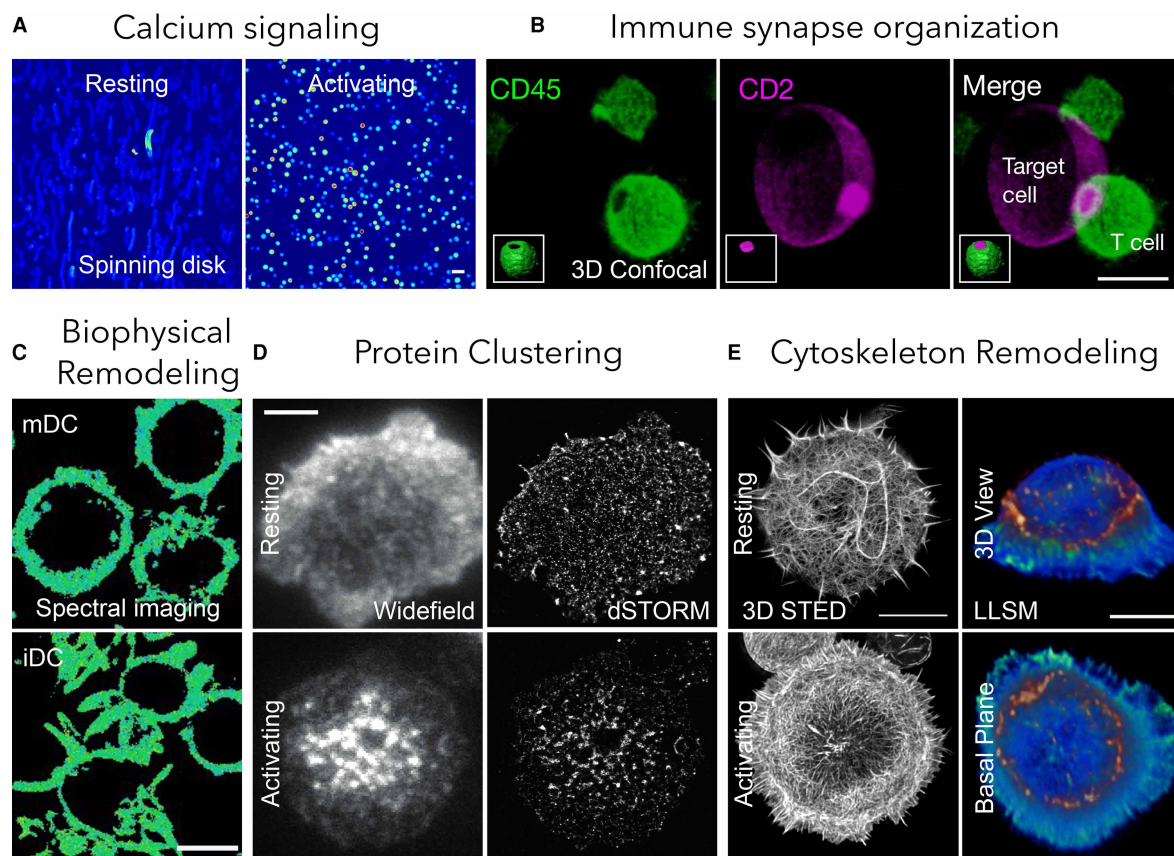
Figure 2. Potential applications of different imaging modalities in immunity.

(**A**) Calcium signaling with spinning disk microscopy [116] where bright spots show calcium flux (scale bar 20 μm); (**B**) 3D immune synapse with confocal microscopy [146] where magenta is CD2 on target synthetic cells and green is CD45 in T cells (scale bar 10 μm); (**C**) biophysical imaging with spectral imaging combined with smart probes [103] where immature dendritic cells (iDCs) show lower membrane fluidity than mature dendritic cells (mDCs) (scale bar 10 μm); (**D**) protein clustering with SMLM [48] (scale bar 3 μm); (**E**) cytoskeleton imaging with STED and LLSM [33] (scale bar 10 μm).

Widefield fluorescence techniques utilize cameras to overcome scanning-induced speed limitations of confocal microscopy, but with reduced axial sectioning abilities. Structured illumination microscopy (SIM) is a widefield super-resolution microscopy technique that uses interference patterns to acquire multiple patterned fluorescence images from varying directions. These sequences then enable reconstruction of super-resolution images. This practically doubles resolution in all dimensions to achieve 125 nm and 350 nm resolutions in lateral and axial dimensions, respectively. In terms of immune signaling, most dynamic imaging studies to date have been performed in 2D [23]. Combination of SIM with advanced analysis techniques such as image correlation spectroscopy has enabled analysis of fast membrane protein dynamics [24]. With instant SIM [25], subsecond cellular 3D imaging has now become possible, but photobleaching problems are still prevalent, preventing long-term imaging.

Confocal, SIM and epifluorescence microscopy are all prone to photobleaching as most of the sample is continuously exposed to laser excitation. To achieve gentler imaging, light-sheet microscopy (also known as selective-plane illumination microscopy) techniques have been developed, which selectively excite fluorophores within the focal plane of the sample. This combines the advantages of TIRFM and off-surface methods to enable 3D imaging with axial sectioning and high contrast due to reduced out-of-focus excitation. However, the difficulty in applying light-sheet microscopy to bioimaging has always involved the complexity of introducing the light sheet into the sample. Given the need for high numerical aperture objective lenses to visualize signaling processes, a variety of complex solutions have been developed including tilted objective lenses [16,26], special sample chambers [27], prisms [28], and reflective surfaces [29–31].

Table 1 Comparison of the available techniques to investigate immune signaling processes (fps: frame per second)

Technique	XY res (nm)	Z res (nm)	Temporal (fps)	Limitation	Advantages	Typical use
Total internal reflection fluorescence (TIRF) microscopy	250	100	100	Only the interface with coverslip	High contrast	Synapse formation, single-molecule dynamics
Variable angle (VA)-TIRF microscopy	250	10	100	Only the interface with coverslip	Z resolution++	Cell topography
Confocal	250	500	20	Photobleaching	Simplicity	Cell structure, interactions
Airyscan	125	250	5	Photobleaching	Resolution	Cell structure, interactions
Stimulated emission depletion (STED) microscopy, ground state depletion (GSD) microscopy	20	50	1	Toxicity, Adequate dyes	Instant super-resolution	Live cell super-resolution, Combination with single molecule
Instant structural illumination microscopy (SIM)	125	250	100	Photobleaching	Speed, resolution	Dynamic cell–cell interactions
Lattice Light-sheet microscopy	250	500	100	Setup complexity	Gentle and fast imaging	Dynamic cell–cell interactions
Lattice SIM	125	250	20	Setup complexity	Gentle and high resolution imaging	Dynamic cell–cell interactions
Single molecular localization microscopy (SMLM)	15	15	-	Chemically fixed cells, Elaborate data analysis	Axial and lateral resolution	Local and global distribution of molecules
Minimal photon fluxes (MINFLUX) microscopy	2 (20)	2 (20)	- (100 μs)	Chemically fixed cells, Elaborate data analysis	Axial and lateral resolution	Cell structure, interactions, Localization of molecules (tracking)

A major improvement came with the development of lattice light-sheet microscopy (LLSM) that offered optical sectioning (1 μm full width at half maximum sheet thickness) over a large field-of-view (50 μm) [6,32]. This imaging modality achieves high resolution (230 nm xy and 370 nm z, or 150 nm xy and 280 nm z in SIM mode) and high-speed imaging while minimizing phototoxicity and photobleaching effects. Over the past seven years, this technique has developed into one of the most promising techniques for imaging dynamic interactions between immune cells in 3D, for example revealing the topological changes taking place during T cell IS formation [32], how finger-like cellular structures search for antigens [6] and how the actin cytoskeleton facilitates immune activation [33,34] (Figure 2). Its combination with adaptive optics has now enabled dynamic 3D imaging of immune cells in living tissue [35]. Recently, high numerical aperture oblique plane microscopy [36] has been implemented to enable high-resolution (300 nm xy and 600 nm z) single-objective light-sheet microscopy for imaging immune cell interactions [37]. This is currently the most accessible choice for immunology labs building their own microscopes. LLSM for common sample geometries has also become accessible through commercial solutions that can achieve multi-color imaging with 700 nm optical sectioning thickness over a field of view of 30 × 300 μm at a speed of 400 frames per second.

Dynamic diffraction-limited 3D imaging of immune cells has mostly been solved with above introduced techniques, although there are needs in terms of imaging deeper into tissue and simplifying setups for increased accessability. While a great deal can be learned, understanding of immune signaling processes on the molecular scale requires nanoscale resolution, thus the field is in need to combine its efforts with super-resolution imaging techniques while maintaining the high temporal resolution offered by the dynamic 3D imaging techniques.

Super-resolution imaging of immune signaling

Super-resolution imaging modalities paved the road to investigate immune signaling down to individual signal events initiated or mediated by single receptors, kinases, phosphatases, adaptor molecules or other contributing biomolecules. Nowadays commercial availability of super-resolution microscopes facilitates their active

utilization to study cellular processes, such as immune signaling, on the nanometer scale. Common to all super-resolution modalities discussed in this section is the suppression of fluorescence emission from labels coupled to the biomolecule of interest to achieve spatial resolutions far below the classical resolution limit of light microscopy (∼250 nm).

In stochastic and camera-based approaches like (direct) stochastic optical reconstruction microscopy ((d) STORM) [38,39], (fluorescence) photoactivated localization microscopy ((f)PALM) [40,41], points accumulation for imaging in nanoscale topography (PAINT) [42], or ground state depletion microscopy followed by individual molecule return (GSDIM) [43], fluorophores are switched between visible and invisible states (for how this is achieved see ref [44]). Conditions are chosen such that most molecules are invisible and hence omitted from imaging. As a result, average distances between visible molecules become larger than the resolution limit of the microscope. Individual molecules can now be computationally localized with precision only limited by the number of detected photons and background noise [45,46], and typically reach values of a few nanometers. By recording tens of thousands of such images and subsequent localization of all single molecule signals, a super-resolution image of molecular positions can be reconstructed. These molecular maps are often consulted to determine the local and global distribution of molecules on the plasma membrane. Examples range from the TCR [47–49], the adaptor LAT [47,50], the kinases Lck [51,52] and ZAP70 [53], and many others, to the complex interplay of individual components within the TCR [54], BCR [55,56] or NK [57] signaling pathways. Numerous mathematical approaches are currently available for the analysis of localization maps (an overview can be found in ref [44]). For most examples provided, the interface between a functionalized glass and an immune cell was studied by using TIRFM as imaging modality. Beside the gain in contrast, the vicinity of fluorescently labeled molecules to glass allows for using the supercritical angle fluorescence as a parameter for determining the distance of fluorophores to the glass surface with nanometer precision also in the axial direction [58,59]. This combination enabled to study the topography of the immunological synapse at isotropic localization precision below 15 nm [49] (Figure 2).

Recording thousands of frames for super-resolution imaging takes minutes to hours, rendering the observation of biomolecular dynamics challenging [60]. Therefore, most studies are still performed on chemically fixed cells. One possibility to overcome residual motion [61] and potential artifacts described for chemical fixation is to conduct super-resolution microscopy experiments at cryogenic temperatures. Although this approach was not applied to study immune signaling yet, current technical developments and first results look promising for using cryo-super-resolution for the routine detection and characterization of biomolecular assemblies on the sub-nanometer length scale [62].

In contrast with these 'single molecule localization microscopy' (SMLM) techniques, stimulated emission depletion (STED) [63] or ground state depletion (GSD) [64], use a deterministic approach to suppress unwanted fluorescence at specific positions. By combining an excitation laser with a second laser, which switches fluorescence off everywhere but in the center of the activation laser, nanoscopic activation spots are created with sizes much smaller than the diffraction limit of ∼250 nm. Scanning of both lasers over the sample and collecting the fluorescence emission by a point detector allows the reconstruction of a super-resolution image. Although the resolution achieved is in most cases worse compared with SMLM, STED is faster and can be used for live cell imaging, for example, to image directly cytoskeletal actin dynamics during IS formation [33] (Figure 2).

Of note, long before the advent of super-resolution imaging, the high precision in localizing individual signals was utilized in single-particle or single-molecule tracking (SPT, SMT) to extract information about locations, kinetics, dynamics and interactions of individual molecule. The basic concept is to directly observe the motion of individual signals in live cell experiments and to extract the aforementioned information from trajectories generated from localized particles or molecules [65]. High-speed SPT and SMT allowed the detection of plasma membrane compartmentalization via hop-diffusion of MHC class II molecules [66] far below the typical resolution limit of light microscopy. The direct observation of interaction dynamics between TCR and peptide-MHC [67,68], or between TCR and ZAP 70 [69] on the millisecond to seconds timescale was facilitated by tracking experiments. Combining SPT/SMT with single-molecule Förster resonance energy transfer (FRET), multicolor and 3D imaging, is hence a powerful approach for live cell studies, however, with one major caveat: the density of fluorescently labeled molecules needs to be low enough for unambiguous tracking. Only if combined with photo-bleaching, SMT can be applied to characterize the entire population of molecules, as shown for characterizing the oligomeric state of the TCR [70].

Super-resolution techniques immensely contribute to our understanding of immune signaling by identifying spatiotemporal positions of individual key molecules on the nanometer-scale. However, the precise mode of action of these molecules within the signaling process needs to be addressed separately. One parameter gaining increasing attention in the field of immunology is the exertion of or response to mechanical forces [71].

Imaging forces in immune signaling

The structural plasticity of immune cells facilitated by cytoskeletal remodeling has been shown to exert a substantial amount of force on neighboring cells, on the extracellular matrix, and to the components of the IS. It changes the conformation of individual molecules, initiates cluster formation, and alters bond kinetics and biophysical properties such as membrane stiffness. Remodeling of the cytoskeleton, similar to cell spreading and motility, results in cycles of pushing and pulling [72,73], which coordinate and terminate different signaling cascades. To understand the role of mechanical forces, novel techniques have to be used and developed.

While we reviewed techniques to probe mechanical forces elsewhere [74], the field has received significant attention with a plethora of new discoveries. There are several methods available to probe the involvement of tensile load application during immune cell activation. For T cells, with astonishing discriminative power, TCR–pMHC interactions are tested by mechanical load application, leading to specific activation [75]. The kinetic proofreading model, being based on TCR-proximal signaling triggered through TCR–pMHC bond lifetimes, was first used to explain the discriminative power [76], with raising attention on mechanical load application [77]. Single-molecule FRET imaging revealed that lifetimes of TCR–pMHC bonds were significantly altered compared to in solution [67], with cellular dynamics mediated by the actin cytoskeleton as a potential reason. The TCR–pMHC unbinding under force was found to have characteristics of both catch bond [72,78] and slip bond [79] behavior, using standard surface plasmon resonance studies as well as biomembrane force probes, where force is measured through reading out the deformation of cells as a force sensor [80]. Recent discoveries have identified a mechanical force range in the piconewton (pN) regime. Note that a force of 10 pN is equal to the weight of ∼10 T-cells or 1 million corona viruses on a surface and it is sufficient to rupture bonds and alter conformations of proteins. The impact of such small forces on immune cell signaling is described in the following.

Besides the structural dynamics of IS formation [81], to trigger T-cell signaling, single TCRs were strikingly shown to be transported to the center of the IS via the cytoskeleton with resulting shear forces of ∼10 pN measured by optical tweezers [82]. Furthermore, micropipette experiments with TCR-ligand coated beads revealed T-cell signaling in the form of calcium release. Beads, therefore, were retracted after T cell engagement [83]; when beads were moved tangentially with respect to the T-cell membrane activation occurred more likely [82], proving that the direction of applied forces matters [76,77]. In a different set of experiments using deformable substrates it was shown that T cells can sense the elastic properties of their surroundings [84]. This technique, named traction force microscopy (TFM) (for review of the techniques in the context of immune signaling please see ref [74]) enabled the study of pulling forces using micropillar assays [85] and hydrogel bound microbeads [86]. Also for B cells, TFM was used to record force application of cells to their surrounding during the activation process [87], suggesting that there is active sensing between groups of immune cells. Most recently, TFM has been combined with 2D TIRF-SIM [88] to correlate force readout with cytoskeletal remodeling and identified the importance of even individual microvilli for the force-mediated T-cell activation process [89].

To detect forces on the single TCR–pMHC bond level, DNA-based tension sensors were instated, suggesting forces as high as 12 pN for ligand-bound TCRs [90] on glass. In contrast, forces as high as 4.7 pN were measured on gold particles attached to a supported lipid bilayer [91]. Tuning the maximal force via the DNA tension sensor enabled ZAP70 recruitment to the engaged TCRs as a secondary signaling molecule [90]. However, DNA tension sensors are hard to predict for ensemble measurements as it is unclear if they are arranged in parallel or zipper-like configuration. Furthermore, it is difficult to estimate how many molecules are engaged simultaneously. Moreover, the unzipping force is strongly loading-rate [92] and duration of applied force [93] dependent. As an alternative, FRET tension sensors have been established for focal adhesions [94] with the perspective of time dependence studies of TCR — pMHC interaction and involved forces [71].

Imaging biophysics of immune signaling

Cellular biophysical properties such as cellular deformability, stiffness, membrane fluidity, viscosity and tension differ for different cells, cellular states and diseases [95,96]. Such properties play crucial roles in immune cell activation, differentiation, and function, therefore methodologies to accurately measure these properties are

needed. Biophysical properties are usually measured with advanced imaging technologies in combination with smart probes. Membrane fluidity can be measured using environment-sensitive probes whose emission spectra change depending on the physicochemical properties of the environment. Polarity-sensitive probes, for instance, exhibit more blue-shifted emission in rigid membranes compared with fluid membranes. To quantitatively evaluate the membrane fluidity, this spectral shift can be detected by widefield, confocal or super-resolution imaging [97]. Membrane viscosity and tension can be measured with molecular rotors [98] or push-and-pull probes [99] which change their fluorescence lifetime depending on the viscosity or the tension in the environment. These probes require sensitive detectors with single-photon counting capacity (so called 'time-correlated single-photon counting' detectors) attached to imaging units. Measuring cellular stiffness, on the other hand, does not require fluorescent probes. Atomic force microscopy has been used extensively to measure the stiffness of cells [100], however, it is limited by its low throughput (i.e. one cell at a time). Recent real-time deformability technologies rely on microfluidics devices measuring the cell shapes using brightfield light [101]. With this technology, adaptability of the shape as a function of physical resistance is used to obtain the stiffness of thousands of cells in a short time [102].

Immune cells undergo extensive biophysical remodeling during their lifetime which is necessary for their function. A recent example by Lühr et al. [103] using environment-sensitive probes, showed that monocyte-derived dendritic cells change their membrane fluidity and cellular stiffness during cellular maturation (Figure 2). In a similar example, Matias et al. [104] demonstrated that regulatory T-cell differentiation is correlated with biophysical remodeling. Importantly, such biophysical properties can influence the migration potential [105] and signaling potency of immune cells [106], which are crucial factors for immune responses, such as during inflammation and infection [107]. Apart from remodeling their own biophysical properties, immune cells can also sense the biophysical cues from their target cells. A recent example by Tello-Lafoz et al. [108] suggested that biophysical vulnerabilities in cancer cells can be recognized and utilized by immune cells. The stiffness of target cells, for example, is a crucial factor for immune cell recognition and activation [17,109,110].

Biophysical properties of the IS attracted particular attention in the last decade. There is significant evidence suggesting a major role of biophysical properties of IS as regulating factors for signaling [111,112]. Owen et al. [113] showed that the IS has higher membrane order compared with the rest of the plasma membrane. Recent studies suggested that this higher membrane order in the IS serve as a protection mechanism against self-killing [114,115]. Moreover, electrical charge in the IS might also be different from the rest of the PM due to the exocytosis of secretory granules with negatively charged lipids. This difference in charge can help fulfil IS function and, similar to membrane order, protect the IS against its own toxins [115]. Jung et al. [100] showed that stiffness of T cell changes upon IS formation which adds another regulation mechanism for IS activity [109]. Size of the molecules at the IS constitutes an additional parameter in the IS biophysical landscape. Tall molecules are excluded from the tight contact between the immune and target cells, which can be a biophysical regulation mechanism for activation of various immune cells [3,116,117]. Finally, diffusion and aggregation of the molecules at the IS have been suggested as a crucial biophysical mechanism to fine-tune signaling [4,118]. The IS has been an important target for therapies such as immunotherapy and the accumulating evidence on the biophysical aspects of the IS shows that such collective biophysical properties should be considered for therapies targeting the IS [119].

Limitations and common artifacts while imaging immune signaling

While advanced imaging techniques reveal new important and complex details about the immune response, they come with technical, biological, and methodological limitations. In many applications mentioned before, the complexity of the biological systems was kept as low as possible to allow for high-contrast imaging. For example, imaging was performed on cells interacting with 2D surfaces to enable TIRFM, or simple cell–cell interactions in solution were observed using (lattice) light-sheet microscopy. Looking directly at immunological processes occurring in tissue, e.g. lymph nodes, demands new imaging strategies or modifications of existing techniques. Recently, the multiscale reorganization of TCRs during the *in vivo* immune response was imaged in lymph nodes by light sheet dSTORM and SIM [120], demonstrating the suitability of advanced techniques also for more complex systems by combining imaging concepts. Other limitations to overcome are the spatial resolution in dynamic imaging — or analogously — the temporal resolution in super-resolution imaging (Figure 1). While current and future developments will address these challenges, the achieved signal-to-noise

ratio depends ultimately on the photophysics of used organic dyes, fluorescent proteins or other fluorescent markers. For all techniques, the number of detected photons will set the limit for achievable temporal and spatial resolution. In addition, the complex and not fully understood photophysics of fluorophores might lead to misinterpretation, as was recently demonstrated for the nanoscale organization of TCR [48] or Lck [52]. Software taking, for example, the experimentally determined blinking properties of fluorophores into account [121] or elaborate experimental strategies (e.g. by titrating the label [52], temporal accumulation analysis [122] or using spectrally distinct probes [123]) might overcome these artifacts.

Limits are also set for the resolution of mechanical forces, where probes and methods sensing a high dynamic force range are still missing. Also, the ultimate aim of a force read-out for individual molecules within the whole IS will demand the combination of various imaging techniques, as was shown in first attempts by increasing the spatiotemporal resolution [88] or addressing forces in 3D [124].

Another major limitation to overcome is the handling and storage of large amounts of data generated during image acquisition. New concepts to increase the computational power for data analysis will have to be instated. The increasing complexity of microscopy systems and generated data will have to be matched by well trained and skilled operators and machine learning/artificial intelligence-based analysis algorithms, respectively.

Future

Immune signaling has been imaged at high speed [32], high resolution [49], multispectrally [125] and deep inside living tissue [35]. The main challenges in the field relate to breaking the pyramid of frustration (Figure 1) to combine these achievements and push towards real-time super-resolution imaging in 3D, ideally while imaging multiple targets and avoiding phototoxicity/photobleaching. Such capability would enable unprecedented insight into how complex players of the immune system coordinate to sustain basic signaling functions. Furthermore, the increased speed and resolution offer opportunities of being combined with force- and signaling-fluorescence assays to understand how these mechanisms correlate with the organization of cellular components.

Dynamic techniques (<1 s per image) are constantly pushing the spatial resolution limits. Parallelized RESOLFT, which relies on photoswitchable fluorescent proteins, now achieves 80 nm 3D resolution in entire cells at 1–2 frames per second [126]. Light-sheet has previously been combined with RESOLFT to achieve <100 nm sectioning [127] and the recent combination of light sheet with 4Pi microscopy achieves <100 nm sectioning with conventional fluorophores [128].

High-resolution SMLM techniques (<10 nm) have traditionally suffered from particularly slow imaging speed. However, improvements in analysis pipelines through deep-learning approaches [129,130], optimization of imaging parameters [131] and novel labeling strategies [132] make dynamic 3D super-resolution imaging a future inevitability. Notably, the recently introduced MINFLUX [133] offers unprecedented spatial and temporal resolution, which will aid in this endeavor. In addition, minimal emission fluxes reduce the probability of fluorophore photobleaching and hence allow tracking of individual molecules over long time periods. Its potential parallelization, as previously done for STED [134] and RESOLFT [126], holds great potential for future dynamic imaging applications.

Another aspect holding the field back is availability of state-of-the-art techniques and analysis tools. As mentioned earlier, it took ~7 years to develop a turn-key commercial LLSM. Fortunately, the microscopy field has been embracing democratization of designs and low-cost solution to bring new techniques to the masses. 3D printed microscopes [135,136] offer great affordability and flexibility. Deep-learning approaches for analysis and denoising are readily available for implementation on online platforms [137]. Complete low-cost designs are available for confocal [138], widefield [139,140] and SMLM [141].

While dynamic imaging is key, there is much left to be learned from static approaches. Expansion microscopy has recently been used to map out receptors on T cells [12], which has the potential to be applied to most immune cells. The structure of the IS is particularly paramount, where SMLM and expansion microscopy offer great potential in visualizing receptor organization. Nevertheless, it is important to acknowledge and resolve the challenges involved both with fixation [142] and expansion microscopy [143] and an exciting solution may lie in the application of cryo-SMLM that can overcome these limitations [144].

Despite these technical developments, there is something inherently difficult about imaging cell–cell interactions and synapse formation and there are only a handful of studies out there taking advantage of the latest methodologies. This partially stems from the move into dynamic 3D super-resolution imaging being far from trivial. The relatively fast restructuring of the cell membrane and the reorganization of membrane proteins

present a complex challenge involving multiple unanswered questions, even with the availability of high-speed high-resolution techniques. Where exactly is the membrane or glycocalyx? Where are the proteins in relation to the membrane? Is the cell currently signaling? Is the cell under stress? Furthermore, the basic definition of detecting signaling is itself challenging: does it correspond to calcium flux, expression of certain proteins or formation of signaling complexes? Complementary labeling approaches have the potential to define where the interactions occur, which can provide a helpful cue [145], but there is a large scope of applying such labeling or biochemical approaches. Nevertheless, one thing is clear: to fundamentally change our understanding of immune signaling through these new visualizations, it will be necessary to develop novel analytical approaches that can quantify multispectral 3D data and correlate it with the highly dynamic complex topography of the cell membrane.

Perspectives

- Advanced imaging is key for visualizing the spatiotemporal regulation of immune signaling.

- We summarize state-of-the-art imaging methodologies and provide recent examples on how they helped to unravel the mysteries of immune signaling.

- We discuss the limitations of current technologies and share insights on how to overcome these limitations to visualize immune signaling with unprecedented details.

Competing Interests

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

Author Contributions

All authors contributed equally to manuscript writing.

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Abbreviations

FRET, Förster resonance energy transfer; GSD, ground state depletion; LLSM, lattice light-sheet microscopy; SIM, structured illumination microscopy; SMACs, supramolecular activation clusters; SMLM, single molecule localization microscopy; STED, stimulated emission depletion; TCR, T cell receptor; TFM, traction force microscopy; TIRFM, total internal reflection fluorescence microscopy; VA, variable-angle.

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