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The Future of Immuno-imaging – Deeper, Bigger, More Precise, and Definitively More Colorful

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Abstract

Immune cells are thoroughbreds, moving farther and faster and surveying more diverse tissue space than their non-hematopoietic brethren. Intravital 2-photon microscopy has provided insights into the movements and interactions of many immune cell types in diverse tissues, but much more information is needed to link such analyses of dynamic cell behavior to function. Here we describe additional methods whose application promises to extend our vision, allowing more complete, multiscale dissection of how immune cell positioning and movement are linked to system state, host defense, and disease.

Keywords

2-photon; dynamic; imaging; in vivo; multicolor

The immune system is like a fine mechanical watch – there are a large number of parts that must work together to achieve the right result. For the watch, the goal is keeping perfect time, for the immune system it is optimally protecting the host. If the pieces are not machined and assembled properly the watch (immune system) can run too slowly (immunodeficiency) or too fast (autoimmunity / inflammatory disease). There is a delicate balance in the interaction of the watch parts – they must move properly, engage for just the right amount of time, then disengage and move again. Likewise the cells of the immune system must circulate and migrate, find the right cellular partner at the right time, engage for the proper duration, signal effectively, change gene expression, then move once again. Dynamics and positioning are crucial aspects of immune function that need to be described and understood if we are to have an accurate picture of the system and how it carries out its functions.

Like a watchmaker, who uses magnifying lenses to peer at the minute parts of a complex timepiece to check their function, investigators have turned to optical imaging to gain knowledge about the dynamic properties of immune cells in their *in vivo* environments. Over the past decade in particular, intravital 2-photon imaging has provided a wealth of insights into what has come to be called ‘immunodynamics.”[1–3] We have seen how naïve T and B cells move within secondary lymphoid tissues and acquire antigenic information [4–14], the intricate dance of T and B cells at the T/B-border and within germinal centers [15–20] and of developing thymocytes in that organ [21, 22], the reactivation of memory T cells [23–25], osteoclast, platelet and neutrophil mobilization in the bone marrow [26–28],

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as well as the movement of innate and adaptive effectors in tissues such as skin [29–31], liver [32–34], central nervous system [35–37], lung [38, 39], and tumors [40, 41] among others. Migration and local probing behavior of dendritic cells in diverse sites has been examined [42–45]. The role of stromal elements in guiding immune cell migration has been discovered [46], the key contribution of adequate cell-cell adhesion in overcoming the dispersive migratory properties of lymphocytes and permitting effective inter-cellular co-operation has become clear [47], the restricted anatomical domains in secondary lymphoid organs within which some innate and adaptive immune cells migrate while awaiting evidence of host invasion have been revealed [48], and the in vivo operation of chemokines with regards to facilitating the encounter between rare cell populations delineated [49, 50].

While these discoveries have ‘animated’ the field for years, we are still far from where we need to be to link information on molecules, signaling pathways, and gene regulatory events to these descriptive dynamics. New tools and techniques are required to see more for longer in larger volumes. Methods that allow simultaneous tracking of receptor signaling events and cell movement, of cytokine production and the response to these key mediators and of gene activation are essential for connecting dynamic behavior with function and differentiation. Regions of tissues currently inaccessible to our imaging platforms need to be made visible, tracking needs to occur in larger volumes to avoid loss of cells over the time span involved in their progression from resting cells to a differentiated state, many more cell types (including stromal elements such as mesenchymal cells, nerves, and vessels) need to be distinctively labeled for visualization at one time, and resolution must be increased to permit intracellular elements to be monitored. New computational tools must be developed to cope with the vast amount of data that will be generated from imaging more colors, with greater resolution, for longer times, and in larger volumes, including visualization methods that make such complex data understandable to the experimentalist. In this Viewpoint, we briefly describe the evolving methods (Figure 1A–E) that will contribute to overcoming these limitations and how their implementation will provide essential insights into immune function in health and disease.

Challenge #1 – Imaging depth

Even in mice, the main experimental animal used for immune system dynamic imaging, many events that need to be visualized are hundreds of microns to millimeters from the surface of a tissue. Current fluorescence-based intravital imaging techniques can only probe the region near the surface (up to 200–300 microns) in dense lymphoid and other tissues; this is because of the dispersive optical properties of these biological structures. To gain information deep in live tissue by multi-photon imaging, photons carrying input information (excitation) have to be efficiently delivered to the location of interest (focus), and the output information (fluorescence) has to be transmitted back via emitted photons that can be collected and detected by light sensors. Within deeper areas of tissues, scattering and absorption processes will dramatically attenuate the excitation laser intensity that reaches the focal point and the heterogeneous structure of the tissue will distort the light propagation wave front, so the excitation light cannot be tightly focused. Such optical aberration is a major factor leading to degradation of image quality.

Recognizing this limitation, many groups have been working on solutions. Near term, the simplest approach is the use of far-red or near-infrared fluorochromes and fluorescent proteins [51–54]. The longer wavelength of the excitation and emission photons involved in imaging such fluorochromes and proteins allow for better tissue penetration with less scattering or absorption than shorter wavelength photons. A penetration depth up to 1.6 mm in mouse cortex has been demonstrated with the laser wavelength tuned at 1.28 μm and emission in the near infrared (IR) range [55].

A very promising method that is not limited by wavelength in this way involves a technique that is termed adaptive optics (AO) (Figure 1B). Such a method aims to pre-compensate the light wavefront distortion inside the live tissue and allow a maximum amount of coherent laser light to reach the focal point. This approach can dramatically improve imaging contrast [56, 57]. While the speed of the wave front optimization process has been increased significantly, compatible with typical dynamic multiphoton intravital imaging methods, the improvement of signal quality using AO approaches comes with the price of a smaller field of view. This is because the wavefront distortion has to be measured and compensated at different locations inside the heterogeneous tissue sample, a process that takes time both due to the physical properties and software control of the adaptive mirrors employed to control the beam. These limitations will eventually be overcome through development of faster hardware and software. Even with current AO technology, one can now perform highly resolved imaging in small fields deep within tissues that are structurally stable (i.e., bone marrow, skin).

Other new optical approaches that extend the penetration depth even further, to beyond millimeter scale, are also in development. One such technique is ultrasound-guided optical imaging [58, 59], which takes advantage of an acoustic wave. The latter is less sensitive to the tissue medium that scatters the light wave and can be used to guide excitation and emission photons. This method can currently achieve imaging resolution up to 12 μm at a depth of 2 mm [59], making it potentially useful not only for deep animal imaging but for application to the dermis in humans, as one example.

These methods not only promise to allow visualization of objects deeper in tissues than presently accessible, but also the imaging over time of larger volumes than can be presently examined. Because of cell motility, capture of information from a larger volume not only reduces sampling error (more events can be tracked), but also permits cells to be followed over longer time intervals (because they stay within the imaging volume). In combination with hardware improvements that permit faster scanning, more sensitive photon detection, and faster movement of the stage holding the specimen, these advances will provide an enhanced ability to link early and late events during an ongoing response on a per-cell basis throughout a larger range of organ compartments. Ultimately, this will allow for continuous tracking of cells and their fates after asymmetric division and during differentiation [60].

Challenge #2 – Multiplex detection

Immunity is a highly concerted process involving many different cellular and molecular players. For this reason, the present imaging methods that typically involve 3–4 colors per experiment are incapable of revealing many of the elements that play an important role in immune processes. This limitation applies at the macro scale (insufficient diversity of cell types and stromal structures visualized), so that the impact of tissue organization and the many different types of cell-cell contacts is not well appreciated. It is also true at the nano-scale – without an ability to multiplex more extensively, we cannot track both cell behavior and the molecules involved in cell interactions and signaling or gene activity. Yet such co-measurements are critical if we are to link bulk dynamics to the processes of cellular activation and response crucial to immune function. Achieving these goals requires new experimental capability to observe multiple different cellular and molecular players at the same time, in the same sample, and with the same imaging configuration.

The current spectral range of fluorescence detection is from near ultraviolet (UV) to near IR. Considering the intrinsic fluorescent bandwidth of typical chromophores, the number of different fluorophores that can be distinguished within this spectral range is limited using conventional filtration methods, even assuming that an ideal set of labels with well-

separated fluorescent spectra can be employed experimentally. Four methods promise to overcome these present limitations by facilitating the simultaneous detection of multiple components (Figure 1C):

1. The use of multiple lasers tuned to different excitation wavelengths. Employed in the proper way, this allows optimized excitation of a variety of fluorochromes rather than the severe compromise typical of single laser instruments, permitting substantial improvement in detection of labels expressed at low levels and the use of more distinct fluorochromes that in combination would not be well excited with single laser systems [61, 62].

2. The development of new chromophores (synthetic dyes or fluorescent proteins) with fluorescence spectra beyond the current range. For example, more near-IR or IR fluorescent proteins have already been developed in the past few years [51, 63–66], extending the palette available for imaging studies with current microscope systems.

3. The application of spectral unmixing strategies. Rather than using filters to isolate distinct (non-overlapping) regions of emission spectra to identify targets, one can "deconvolve" the entire emitted fluorescent spectrum to identify the target chromophore by its specific emission spectral profile [67]. This requires the chromophore to have a signal strong enough to be split into the multiple spectral windows, each of which captures fewer total emitted photons. One has to find a balance between the spectral precision (more windows) and sensitivity (signal/noise = larger windows), setting a limit to the use of this approach when the cell or molecule of interest can only have a limited overall fluorescent output.

   A related approach is to label each target component with multiple chromophores and modulate the ratio between different chromophores to identify various biological components (spectral painting). This approach has been successfully demonstrated in the application of single molecule mRNA FISH [68] and in Brainbow [69] and similar transgenic animals. Here, the complex emission spectrum from each different color combination provides a signature for that target. This approach can be further enhanced by careful choice of chromophores with distinct absorption spectra permitting selective excitation at different laser wavelengths when using multi-line instruments.

4. The combination of standard wavelength detection methods with other strategies for fluorochrome identification. One well-established approach in single cell imaging is fluorescence lifetime measurement (FLIM) [70, 71] and this has recently been incorporated into multiphoton intravital imaging [72]. In this method, special detectors and software statistically characterize the time interval (on a nanosecond scale) between the excitation and emission events for each chromophore molecule. Many spectrally-overlapped chromophores have very distinct fluorescence lifetimes, so this technique can be used to add another dimension to the "identity space" of these labels. With the newest generation of high sensitivity detectors on commercial microscopes, implementation of FLIM is becoming an available tool for intravital multiphoton imaging.

A final area in which substantial progress is needed in the area of multiplex detection is in the creation of optimized probes for the analysis of intracellular signaling, molecular localization, or gene expression. A few intravital studies have characterized such events, most involving dye-based calcium sensors [13, 73], in vivo staining [74], fluorescent chimeric proteins [75, 76], and fluorescent gene reporters [77–80]. The calcium studies are limited by the leak rate of the sensor dyes to just an hour or two after cell transfer, the chimeric proteins are both hard to detect and the analysis suffers from artifacts of optical

resolution limitations and signal intensity differences in the axial dimension, and the gene reporters produce cytoplasmic proteins whose lifetime greatly exceeds that of cytokine transcripts, thus failing to provide a properly time-resolved record of gene activity that can be linked to cell dynamics [81]. To make progress in these areas, these limitations must be overcome. New fluorescent proteins have been developed [82] for the generation of optimized FRET sensors that can detect signaling events such as calcium elevation, MAPK pathway activation, and the like and should allow creation of genetically-labeled cells whose signaling can be tracked without the present post-transfer time limitation. The improved sensitivity of newer instruments will permit better detection of chimeric proteins expressed at close to physiologic levels and some of the methods described below and by Sixt in this issue will enhance axial resolution that presently limits such analyses. The creation of a new generation of genetic reporters producing destabilized fluorescent reporters, or secreted versions of such proteins, will improve the temporal connectivity between appearance of these labeled proteins and the underlying genetic activities of the cell of interest [83]. Together, such tools will allow investigators to link molecular events with cell behavior.

Challenge #3 - Faster imaging

The complexity of biological systems not only exists in the space domain, but also in the time domain. Cellular and molecular dynamics have multiplex temporal features ranging from femtoseconds to hours. Both the sampling speed and length of image collection of present instruments and their linked computers dictate the time resolution that can be achieved.

To date, the predominant scheme for sample illumination is point scanning, which limits information collection rate due to the time needed to move the beam over the entire x-y dimensions of the imaging field. A major breakthrough in the past few years allows for parallel illumination of biological samples, sometimes in three dimensions. In these methods, a modified optical system illuminates a selected plane rather than a single point inside the biological sample, with the fluorescence from the whole plane detected by a wide field imager (Figure 1D). For example, light sheet microscopy [84–86] uses one lens to create a narrow sheet of laser focus inside the tissue, and another objective is used to acquire fluorescent images at the perpendicular direction. Bessel beam-based light sheet microscopy [87] further improves the spatial resolution especially in the axial dimension. Another temporal focusing scheme [88, 89] modulates the excitation laser pulse width so that the multiphoton excitation is confined in a sheet-like region. Although the temporal focusing method is limited to non-linear excitation, it brings the convenience of using only a single objective lens with less spatial restriction for sample arrangement.

Challenge #4 - Higher resolution

In principle, the resolution of an optical microscope is limited by the optical diffraction, in the range of a few hundred nanometers with a lens of the highest possible numerical aperture (NA). In practice one has to find a balance between the working distance of the lens and NA, preventing use of the best resolving lenses for most intravital imaging purposes. In addition, the optical aberration inside tissues also degrades resolution, so methods to correct such aberrations, like the AO approaches discussed above, are needed to achieve near-diffraction-limit resolution deep inside tissues.

During the past decade, however, there have been a number of exciting breakthroughs that allow biological samples to be imaged at a resolution far beyond the optical diffraction limit (Figure 1E). These novel approaches can be generalized into two types: one type takes advantage of single molecule location measurement, such as PALM (Photoactivated localization microscopy), FPALM (Fluorescence photoactivated localization microscopy),

and STORM (Stochastic optical reconstruction microscopy) [90–92]; the other creates sub-diffraction excitation patterns, such as STED (Stimulated emission depletion) [93] and Structured Illumination [94]. These new techniques are poised to reveal detailed molecular and structural information inside live tissues. Although to date most applications are with relatively flat cells in culture systems, some exciting recent developments have begun to incorporate such schemes into intravital imaging scenarios [95], permitting the detection of subcellular events with improved precision, at least close to the surface of various tissues [96].

Challenge #5 – Data capture and processing

All the above advances will help collect more information on the immune system in situ, but without the proper means for data analysis, we will make little progress in understanding things. A new generation of software tools is needed to better analyze the image data, preferably allowing more to be done automatically and in an unbiased manner than is presently possible [97]. Some progress has been made in this arena over the past several years, both by commercial software vendors and academic centers, but much more is needed. Optimized algorithms are required to handle the much larger number of objects to be tracked when the imaging volume is increased and more cell types are visualized in more colors, for both analytic purposes and for display of the underlying cell movements; embryologists have made substantial progress in this direction [86, 98] and their methods need to be adopted (and adapted) by immunological imaging experts. Progress is also needed to address specific issues in molecular imaging, which is highly sensitive to depth-related image intensity artifacts. Some schemes to deal with such problems have already appeared [75, 79] but as the field embraces molecular imaging going forward, further improvements will be crucial. Other analytic tools that have been introduced to handle the analysis of cell movements into and out of defined volumes such as germinal centers or to measure synaptic dimensions[99, 100], provide a display in two dimensions out of four or more parameters such as time, speed, directionality, and distance from a defined site. These parameters greatly aids in understanding chemosensing behavior (Lämmermann and Angermann, unpublished), differentiate random walk from other migratory behavior [101, 102], and evaluate contact times between two cell types [103]. These data sets may then be used to derive mathematical models of complex cellular behavior, which by iterative processes can be refined and may allow for prediction of biological outcomes that can be tested in in vivo models [102, 104–110]. Finally, the field will need to move away from the maximum projection the two-dimensional movies that are currently used to three-dimensional displays that allow a better appreciation of depth in the full imaging volume.

Conclusion

Here we have very briefly surveyed some of the emerging techniques that will aid our probing of the dynamic behavior of the immune system going forward. They will allow a larger portion of an organ to be examined, many more elements to be tracked simultaneously, cell analysis to be combined with molecular imaging, and cell function linked to dynamic behavior. An essential point to emphasize, however, is that while such imaging can be revealing in its own right, it is most valuable as a part of the larger fabric of immune investigation; dynamic imaging data need to be properly associated with information gathered by other means, such as static tissue imaging [111, 112], flow cytometry, ex vivo assessment of lymphocyte activity and polarization, genetic and epigenetic studies, and overall measurements of systemic immunity and host resistance. Only through integration of information garnered using the full range of methods available to the field can we develop a comprehensive model of immune system behavior, in which events on the micro and macro scale are linked to the meso-scale dynamics of individual

cells that are the present focus of imaging analysis. With the increased depth and breadth of analysis we anticipate from rigorous application of the methods reviewed here, we are confident the future is, not to put too fine a point on it, “bright.”

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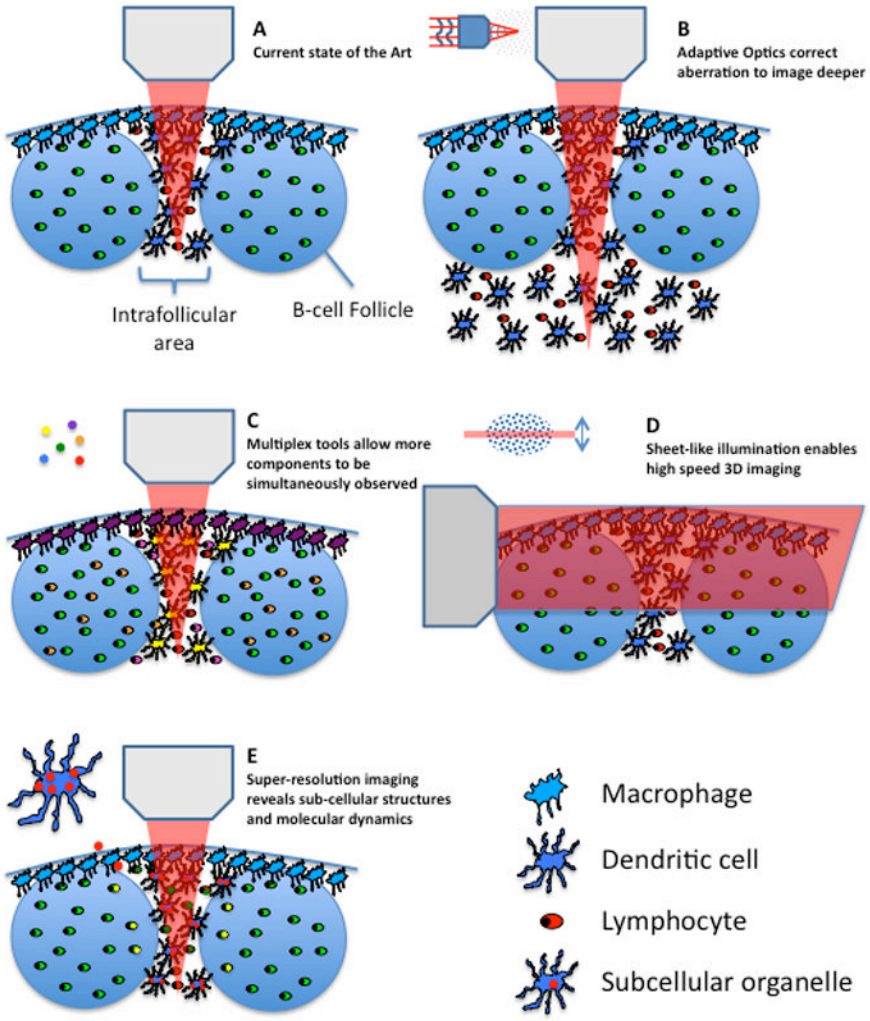

Figure 1. Overview of improved imaging modalities

(A) The current state of the art in imaging the interfollicular area and B-cell follicles of a lymph node is shown. (B) Improved imaging depth by means of adaptive optics is shown. (C) Detection of multiple different signals by means of multiplexing tools such as multiple lasers or new chromophores is shown. (D) High-speed 3D imaging using sheet-illumination is shown. (E) The visualization of sub-cellular compartments using super-resolution imaging techniques is shown.