



New dimensions in intravital multi-photon imaging of immune reactions

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ABSTRACT

In the last two decades intravital multi-photon imaging has become a central tool to investigate cellular and molecular dynamics of immune reactions *in vivo*. Currently, challenges in exploiting the full power of this technology include limitations on the number of simultaneously detectable parameters as well as in expanding the acquisition in time and space. Here we discuss technological advancements developed in order to overcome these challenges and focus on the example of germinal center reactions as multi-parametric immunological processes evolving over a time course of days and weeks.

Introduction

Over one hundred and thirty years ago, 1892, Ilya Metchnikoff put starfish larvae under a microscope, pricked them with thorns to introduce foreign material, and observed cells accumulating at these sites. Looking more closely, he noticed material was accumulating inside the cells, and he termed this phagocytosis. This is generally accepted as the foundation of cellular immunology. A century later, it is now clear that humoral immunity also involves complicated interactions between many cellular partners.

Although we know vastly more about the mechanisms by which immune responses develop, some of the basic questions remain the same. How do the cells interact with each other in living tissues? What are the functional effects of these interactions on the various cellular components? How do all of these processes evolve over time, and lead to productive - or failed - immune responses? How do pathogens and malignancies seek to manipulate these processes? How do they differ among tissues? To understand all of these processes *in vivo* necessarily starts with observing them. As these processes are affected by factors such as temperature, relative cell frequency, anatomical positioning of cells relative to each other, oxygen tension, and the concentration of various host- and pathogen-derived signaling molecules and metabolites, among countless other factors, a comprehensive picture is only obtained in living tissue. This is especially true of the germinal center reaction, which is responsible for generating high-affinity antibodies necessary for effective humoral immunity.

Germinal center reactions

After infection, B cells produce antibodies to neutralize and opsonize pathogens. Interestingly, over time, the affinity of these

antibodies for their target antigen increases¹, a process known as affinity maturation. This has been shown to be due to repeated rounds of somatic hypermutation and affinity-based selection occurring in a specialized region of secondary lymphoid organs, called the germinal center (GC) and illustrated in Figure 1²⁻⁴. The germinal center itself is divided into two zones that are each responsible for one of the above functions: to a first approximation, GC B cell division occurs in the dark zone, and affinity-based selection occurs in the light zone⁵⁻⁸.

After B cells recognize their antigen through their B cell receptor (BCR) a fraction of activated B cells relocate to the interfollicular zone, where they present antigenic peptides on MHC II to T helper (Th) cells⁹⁻¹². Upon recognition of cognate antigen on an activated B cell, the T cell signals to the B cell through several signaling pathways (B7, CD40-

CD40L, etc.) leading the activated B cell to proliferate; further successful antigen presentation and T follicular helper (T_{FH}) cell signaling licenses the activated B cell to become a GC B cell and migrate to the GC in the center of the B cell follicle¹³⁻¹⁵.

Intravital imaging has demonstrated that GC reactions are highly dynamic and involve the interactions of multiple cells types¹⁶⁻¹⁸. The GC reaction starts with the uptake of antigen by the B cell receptors of GC B cells from other, lower-affinity B cells^{19,20} or from an extensive network of specialized stromal cells called follicular dendritic cells, which retain antigen in the form of immune complexes on their surface for months^{21,22}. After GC B cells have taken up antigen with their BCR, the antigen is internalized and degraded via the proteasome, and peptides are presented on their major histocompatibility complex (MHC) II. If the

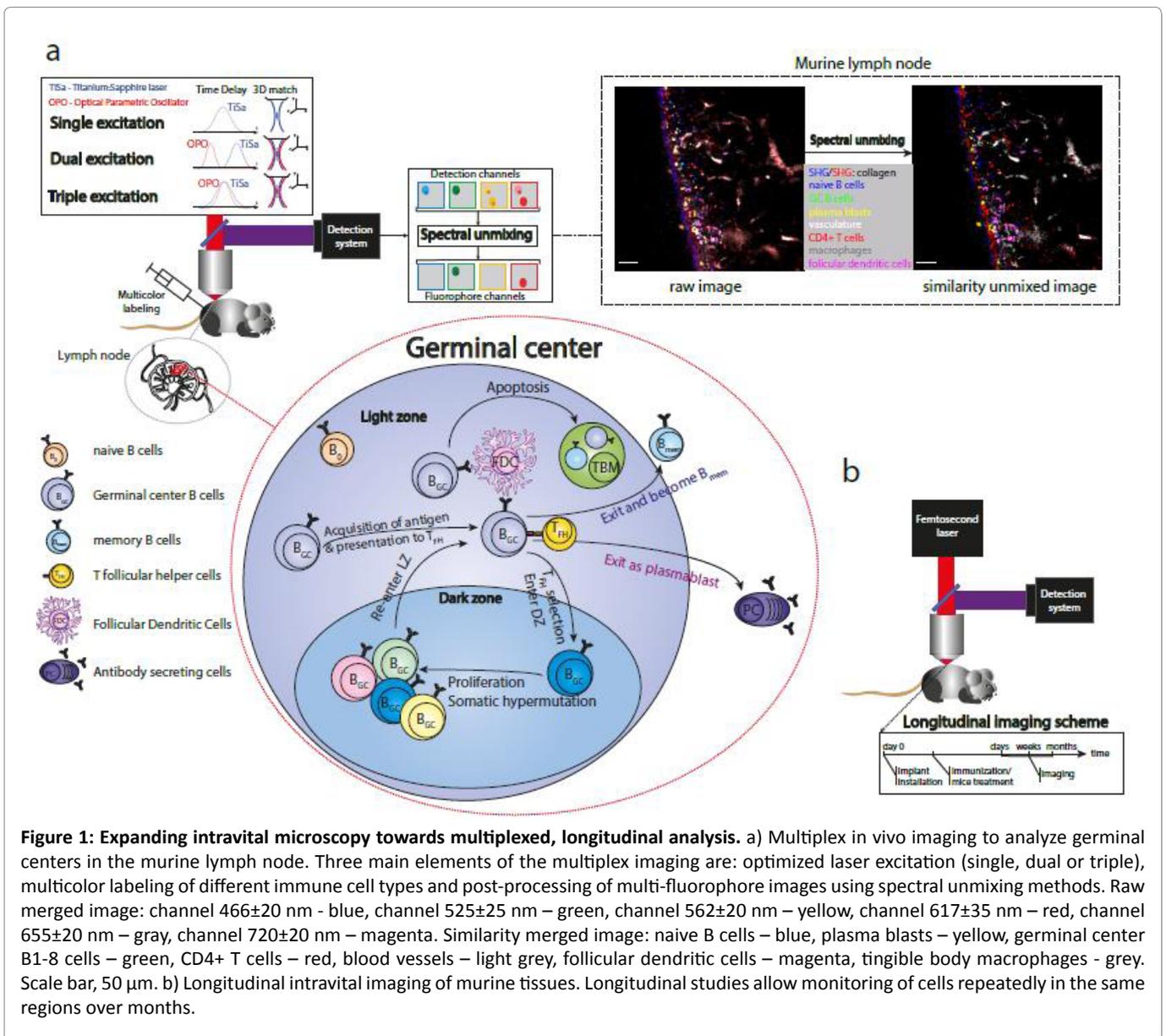


Figure 1: Expanding intravital microscopy towards multiplexed, longitudinal analysis. a) Multiplex in vivo imaging to analyze germinal centers in the murine lymph node. Three main elements of the multiplex imaging are: optimized laser excitation (single, dual or triple), multicolor labeling of different immune cell types and post-processing of multi-fluorophore images using spectral unmixing methods. Raw merged image: channel 466±20 nm - blue, channel 525±25 nm – green, channel 562±20 nm – yellow, channel 617±35 nm – red, channel 655±20 nm – gray, channel 720±20 nm – magenta. Similarity merged image: naive B cells – blue, plasma blasts – yellow, germinal center B1-8 cells – green, CD4+ T cells – red, blood vessels – light grey, follicular dendritic cells – magenta, tingible body macrophages - grey. Scale bar, 50 µm. b) Longitudinal intravital imaging of murine tissues. Longitudinal studies allow monitoring of cells repeatedly in the same regions over months.

presented antigen is recognized by a cognate T_{FH} cell, the T_{FH} cell signals to the GC B cell through several pathways (CD40-CD40L; ICOS-ICOS-L, SLAM, etc.), licensing the GC B cell to engage the dark zone gene program and divide. The dark zone program is also responsible for somatic hypermutation, by inducing the cytosine deaminase AID, which deaminates cytosine to uracil at heavily transcribed loci, especially the Ig locus^{23,24}; repair of this by uracil-N-glycosylase and error-prone DNA repair introduces further mutations^{25,26}. The newly mutated BCRs are expressed on the surface of the GC B cell, where these B cells will compete for antigen against the remaining GC B cells. Significant evidence indicates that the limiting factor in the GC reaction is T cell help²⁷⁻²⁹. To ensure that the presented antigens are representative of the presently-encoded BCR and not retained peptide:MHC from previous "versions" of the BCR, surface peptide:MHC complexes are degraded at late stages of the dark zone program, prior to re-entry into the light zone³⁰. Many repeated rounds of antibody diversification and affinity-based selection lead over time to dramatic improvements in antibody affinities and pathogen neutralizing abilities. GC B cells that do not receive T_{FH} signals die by apoptosis and are rapidly phagocytosed by tingible body macrophages³¹. Cells exit the germinal center either as memory B cells, or as plasma blasts^{32,33}.

Tools for intravital multi-photon imaging of immune responses

The methods used to visualize immune reactions have certain limitations which restricts the questions that can be experimentally answered. For instance, to visualize processes with cellular resolution requires three-dimensional microscopic fluorescence imaging, with a time resolution sufficient to capture migrating cells. To image deep within tissue while minimizing toxicity requires expensive pulsed lasers for two-photon excitation. Upon activation of lymphocytes, they rapidly undergo several cell divisions. If the cells are labeled with a fluorescent dye, it will be rapidly diluted out within a few days. This means that to be identified more than a few days after transfer, cells must be labeled with a genetically encoded fluorophore.

Almost all of the genetically encoded fluorophores presently in use are derived from jellyfish (*Aequorea victoria*) or various corals (*Discoma* sp, *Entacmaea quadricolor*)^{34,35}. These proteins native to aquatic invertebrates did not evolve to maximize their molecular brightness or photostability upon laser illumination in mammalian tissue, and thus all of the useful genetically encoded fluorophores have required much *in vitro* selection and optimization to make them more useful. Further, all of these fluorophores are restricted to a small fraction of the available optical spectrum, from 400 to 660 nm. Unfortunately, the visible spectrum in which

the fluorescent proteins fall is suboptimal for imaging, as shorter wavelengths are scattered more. Increasing the emitted wavelength to the near-infrared leads to a dramatic decrease of scattering, hence, permitting more sensitive imaging much deeper in tissue. Recently, there has been progress in developing near-infrared fluorescent proteins derived from various bacterial phytochromes, but these are still at a relatively immature state of development regarding brightness, photostability and the *in vivo* availability of necessary cofactor, compared to the visible fluorescent proteins³⁶. To identify multiple cell types - as in the germinal center, which contains GC B cells, recirculating naive and memory B cells, and Tfh cells, among others (Figure 1) - requires each to be unambiguously labeled with a genetically encoded fluorescent reporter. Hence, a broad palette of fluorescent proteins is required spanning a large range of emission wavelengths, which in turn generates its own technological challenges, as discussed in the following section.

To understand the cellular and molecular diversity of the immune system represents one of the fundamental challenges for modern imaging techniques³⁷. In this respect, intravital two-photon microscopy, being the leading method to monitor cellular and molecular dynamics in genuine tissue environment, is still limited with respect to multiplex detection and simultaneous visualization of many diverse cellular players of immune processes, i.e. maximally four parameters. There are three main elements of multiplex simultaneous detection *in vivo*: a broad palette of fluorescent markers (synthetic dyes or fluorescent proteins), optimized excitation laser sources and, finally, effective spectral unmixing (Figure 1a)³⁸. The choice of fluorescent markers depends on scientific questions³⁴ but the more markers are multiplexed to label cells, the more information can be simultaneously extracted from one experiment. The development of new fluorophores expands rapidly towards the near-IR/IR range allowing deeper tissue imaging and higher quantity of multiplexing^{36,39,40}. Various excitation schemes were used to detect multiple cellular markers in different organs and tissues. Characterization of six cellular components was illustrated by using sequential single laser excitation in a brain glioblastoma tumor of triple transgenic mice⁴¹. The effect of dual near-IR/IR excitation was shown by imaging three fluorescent proteins in the brain stem of transgenic mice⁴², and five cellular signals were detected within an exogenously grown tumor in immunodeficient mice⁴³. The dual two-photon excitation is achieved by matching foci volumes of both near-IR and IR laser in space. Hence, the molecules within the sample can be excited either with two near-IR or with two IR photons. The power of triple two-photon excitation was demonstrated to intravital visualize three chromophores and second harmonic generation (SHG) in fly embryos⁴⁴, and seven

fluorophores in distinct cellular and tissue compartment in murine lymph nodes³⁸. The triple two-photon excitation scheme is based on a wavelength mixing technique^{45,46}, which implies temporal synchronization of pulse trains from two lasers with the same repetition rate as well as spatial overlap of their foci volumes (Figure 1a). The SHG can be detected from a tissue with ordered structure, i.e. collagen fibers, where the emitting photons have twice the energy of the initial photons. The triple excitation scheme allows simultaneous imaging of more fluorophores with broader spectral range as compared to the single and dual excitations. Although the broad palette of chromophores can be visualized by the optimized excitation scheme, the identification of different cell types is still hindered by the spectral overlap of fluorophores emitting at similar wavelengths. For this reason, spectral unmixing is vital for unambiguous discrimination of multiple fluorophores. Among all methods, linear unmixing is a classical and widely used method, which is implemented in commercial as well as in open-source software (Fiji/ImageJ PlugIn of J. Walter). However, because of its analytical nature, the linear method does not allow the separation of more fluorophores than there are detection channels^{47,48}. As an alternative, similarity (SIMI) unmixing represents a powerful algorithm, where color separation is performed based on the similarity of mixed fluorophores with spectral signatures of known chromophores³⁸. Moreover, SIMI can be used as a complementary tool to linear unmixing, because it is not limited by the linear unmixing condition between the number of fluorophores and the amount of detection channels.

Imaging clonality

As depicted above in Figure 1, the germinal center reaction generates high-affinity antibodies by repeated rounds of diversification and affinity-based selection. Several clever combinatorial methods have been developed to label cells with different combinations of fluorescent proteins, enabling closely apposed cells and cellular processes to be distinguished⁴⁹. This has been used to follow the clonality of germinal centers, revealing that anatomically nearby germinal centers within one lymph node may have very different compositions and be clonally independent of each other⁵⁰. To distinguish multiple clonal variants of B cell, in addition to antigen-specific Tfh cells and wild-type control B cells, requires an unmixing algorithm like the one described above (Figure 1a).

Imaging tissue and cellular function

When techniques for intravital imaging were first developed, there were relatively few fluorescent reporters of protein function, limiting the observations to quantifications of cellular movement and interactions. One promising technology that can provide a link from cellular

dynamics to cellular function is Förster Resonance Energy Transfer (FRET). This technology uses the phenomenon of non-radiative (via dipole-dipole interaction) energy transfer that converts the excitation from a fluorophore serving as a donor to the fluorescence of another chromophore or acceptor in the range of 1-10 nm. Based on FRET technology many methods have been developed using different relationships of donor-acceptor intensities, excited state lifetimes, and emission anisotropy. The methods based on intensity relationships require an accurate reference for acceptor-free donor signals and correction for spectral overlapping of donor and acceptor signals. The techniques based on determination of fluorescence lifetimes are one of the most direct ways to quantify FRET, allowing to avoid effects of photobleaching and differences in signal-to-noise-ratio of donor and acceptor molecules⁵¹. Emission anisotropy measured at different polarized excitation provides information of rotational diffusion being sensitive to size, shape, orientation and motion of the molecules. A bright illustration of intravital application of FRET technology has been shown using Ca²⁺ imaging of brain in transgenic mice CerTN L15, which contain a troponin-C FRET-based calcium biosensor⁵²⁻⁵⁴. Referring to cells of the immune system, FRET-based indicators of intracellular calcium have been used to measure lymphocyte activation⁵⁵. Many further reporters have been developed, indicating such parameters as voltage⁴⁹, caspase-3 activity³¹, the concentration of molecules including H₂O₂, lactate, glutamate, and the location within the cell of transcription factors such as NFAT, Foxo1, or relB. To multiplex various functional reporters necessarily requires that they each use easily distinguishable fluorescent proteins.

Longitudinal intravital imaging

Although acute inflammation is detectable within seconds, with leukocytes moving on the order of one to three cell diameters per minute, immune reactions occur over a time scale of weeks. This imposes significant challenges for observational studies, as individual cells must be followed with a time resolution sufficient to follow and track the cells over time. Imaging sessions can be maintained for a period of several hours, but this is still two to three orders of magnitude too short to observe the evolution of high-affinity antibody responses. To visualize the immune response in its entirety will require methods for repeated longitudinal imaging of the same lymph node. However, this is challenging, as imaging of lymph nodes requires their surgical exposure, which is not considered a survival procedure; even if it was, the repeated surgical exposure would cause significant, prolonged, recurrent inflammation in the lymph node, confounding experimental observations. Longitudinal imaging methods have been well established for imaging of the brain⁵⁶, spinal cord, orthotopic malignancies, and bone marrow⁵⁷

“Longitudinal intravital imaging of the femoral bone marrow reveals plasticity within marrow vasculature.” by Reismann D, Stefanowski J, Günther R, Rakhymzhan A, Matthys R, Nützi R, Zehentmeier S, Schmidt-Bleek K, Petkau G, Chang HD, Naundorf S, Winter Y, Melchers F, Duda G, Hauser AE, Niesner RA. in *Nature Communications*, 2017 Dec 18;8(1):2153. doi: 10.1038/s41467-017-01538-9.), but to date there remains only one method for longitudinal imaging of lymph nodes: orthotopic transplantation to a superficial site, permitting direct observation through the skin. This method, although relatively unexplored, has the potential to vastly increase the scope of intravital imaging of immune reactions – longitudinal imaging is a prerequisite for any sort of long-term labeling or manipulation (Figure 1b).

The goal of longitudinal imaging is to follow the cells from one imaging session to another. The development of photoactivatable proteins - photoconverting a dark fluorescent state of a chromophore to a bright state in response to the light with a specific wavelength or intensity - made this possible, as cells can be individually photoactivated and observed as long as the fluorescent protein remains visible; this was used to label, isolate, and track GC light zone and dark zone cells for shorter periods of time⁵⁸. However, this remains challenging, as photoactivated proteins are degraded due to normal protein turnover, and diluted out by cell divisions. The rapid division of GC B cells, with a cell cycle time of 6-12 hours¹⁶, makes GC cells impossible to track for longer than a day or two using directly photoactivatable fluorescent proteins.

The recent development of methods for optical induction of genetic changes, such as photoactivatable Cre recombinase⁵⁹⁻⁶¹ will make it easier to irreversibly label imaged cells and their progeny, permitting the daughter cells to be tracked over the course of weeks. This too is still a work in progress, with the necessary tools still being optimized for the available imaging and activation methods; the longitudinal imaging necessary for such long-term tracking is itself still under development.

Outlook: optical manipulation

The development of optically activatable systems has enabled microscopy to switch from a purely observational method to one capable of experimental manipulations. The above-mentioned systems for optical activation of Cre recombination should permit the deletion or activation of any arbitrary gene program in an optically targeted population of cells⁵⁹⁻⁶¹. Similar systems have been generated to optically induce or silence transcription at a given genetic locus, if reversible changes are sought⁵⁹⁻⁶³. When longitudinal imaging of lymph nodes is fully integrated with optical methods for labeling and manipulating cells,

this will enable immunologists to fully use the optogenetic tools exploited so productively by neuroscientists over the last ten years.

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