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Two-Photon Imaging of Microbial Immunity in Living Tissues

Jasmin Herz, Bernd H. Zinselmeyer, and Dorian B. McGavern

National Institute of Neurological Disorders and Stroke, The National Institutes of Health,
Bethesda, MD 20892, USA

Abstract

The immune system is highly evolved and can respond to infection throughout the body. Pathogen-specific immune cells are usually generated in secondary lymphoid tissues (e.g., spleen, lymph nodes) and then migrate to sites of infection where their functionality is shaped by the local milieu. Because immune cells are so heavily influenced by the infected tissue in which they reside, it is important that their interactions and dynamics be studied *in vivo*. Two-photon microscopy is a powerful approach to study host-immune interactions in living tissues, and recent technical advances in the field have enabled researchers to capture movies of immune cells and infectious agents operating in real time. These studies have shed light on pathogen entry and spread through intact tissues as well as the mechanisms by which innate and adaptive immune cells participate in thwarting infections. This review focuses on how two-photon microscopy can be used to study tissue-specific immune responses *in vivo*, and how this approach has advanced our understanding of host-immune interactions following infection.

Keywords

two-photon microscopy; virus; immunity; infection

Microscopy Toolbox

Two-Photon Laser Scanning Microscopy

Pioneered in 1990, two-photon laser scanning microscopy (TPLSM) has vastly improved our understanding of biological systems and their cellular constituents by permitting real-time deep tissue imaging (Denk et al., 1990). Today, the standard two-photon microscope is equipped with a titanium-doped sapphire (Ti:sapphire) laser (Moulton, 1986) that generates near-infrared light with pulse widths of 100–150 femtoseconds and repetition frequencies of ~80 MHz (Bullen et al., 2009; Zinselmeyer et al., 2009). To generate light for two-photon microscopy, Ti:sapphire oscillators are normally pumped with a green laser (e.g., 532-nm diode-pumped solid-state laser). The oscillator then generates ultrashort pulses of near-infrared light with a large, highly tunable bandwidth (ranging from 700 to 1,200 nm). Fluorophores or fluorescent proteins in tissues under investigation are excited when they absorb two photons nearly simultaneously as predicted by Goeppert-Mayer (1931) and first observed by Kaiser and Garrett (1961). Two-photon excitation is achieved by using an objective lens to focus near-infrared light onto a small point (i.e., a diffraction limited volume) within the sample. This ensures that the probability of a fluorophore absorbing two photons is highest at the focal plane. Thus, one advantage of using TPLSM is that excitation above and below the plane of interest is largely eliminated. Other advantages include the fact that infrared light is less susceptible to scattering as it penetrates tissue, and reliance on

two-photon absorption minimizes overall tissue background. The net result is that TPLSM enables investigators to perform imaging experiments deep into tissues of interest with minimal phototoxicity. The exact depth of imaging depends on the properties of the tissue being examined, but imaging depths of greater than 1 mm have been reported in the brain (Kobat et al., 2011). Another consideration when using TPLSM is the speed of acquisition. Two-photon microscopes can be equipped with resonant (rather than conventional) scanners that operate at frequencies of up to 16 kHz. This addition markedly enhances acquisition speeds, which improves time resolution and facilitates examination of high speed events such as Ca^{2+} flux (Wei et al., 2007) or cells moving through the blood (Zinselmeyer et al., 2008).

Generating Light

Before conducting a TPLSM experiment, it is important to have a set of fluorescent probes, dyes, proteins, etc. available that are compatible with each other and will yield sufficient signal when excited by two-photon light. Generally, the spectra and brightness for fluorophores excited by two-photon light are not easily predicted by their one-photon properties (Drobizhev et al., 2011). Some absorption spectra are identical when excited by one- versus two-photon light, whereas others differ considerably. Thus, it is important to define optimal two-photon excitation wavelengths empirically before conducting TPLSM experimentation. Absorption spectra and relative brightness measurements are already available for many commonly used fluorophores (Albota et al., 1998; Makarov et al., 2008; Drobizhev et al., 2011). For example, a recent study defined the two-photon properties of 48 different fluorescent proteins (Drobizhev et al., 2011). Excitation wavelengths and brightness measurements are provided for each fluorescent protein, which is an invaluable resource for those conducting TPLSM experiments.

Another consideration for TPLSM experiments is the physical properties of the fluorophores used to generate light. An ideal TPLSM fluorophore should be nontoxic, bright, photo-stable, and have a narrow emission curve to limit cross-channel contamination. For example, cell tracker probes such as blue-fluorescent 7-aminocoumarin (Cell-Tracker Blue CMAC), carboxyfluorescein succinimidyl ester (CFSE), orange-fluorescent tetramethylrhodamine (Cell-Tracker Orange CMTMR), and red-fluorescent CellTracker Red CMTMPX are commonly used to label and monitor cells by TPLSM. The advantage of these cell tracker compounds is that they are very bright and permit multicolor imaging experiments (Zinselmeyer et al., 2009). The downside is that cells must be extracted from a tissue of interest, labeled *ex vivo*, and then adoptively transferred into another recipient prior to TPLSM imaging. This eliminates the possibility of studying an undisturbed cell (prior to extraction) in its natural environment. Another disadvantage is that cell tracker dyes will decrease in intensity by 50% following each cell division. Thus, rapidly dividing cells like T lymphocytes will become more difficult (and, eventually impossible) to track as they divide.

Fluorescent proteins provide an excellent alternative to the use of cell tracking dyes. The 2008 Nobel Prize in Chemistry was awarded for the discovery and development of green fluorescent protein (GFP), which is derived from the jellyfish *Aequorea victoria* (Shimomura et al., 1962; Prasher et al., 1992). From this parent protein many commonly used derivatives have been generated such as cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), etc. Since the discovery of GFP, other fluorescent proteins have been extracted from corals, which have further diversified the color palette available for TPLSM experiments. DsRed was obtained from the coral *Discosoma* (Matz et al., 1999) and was later used to generate derivatives such as monomeric orange fluorescent protein (mOFP), monomeric red fluorescent protein (mRFP), mCherry, etc. (Shaner et al., 2004). Another recent addition to the family of useful fluorescent proteins is monomeric teal fluorescent protein (mTFP), engineered by using tetrameric CFP from *Clavularia* coral as a

starting point (Ai et al., 2006). The major advantage of mTFP is its photostability and high quantum yield. It is approximately twofold brighter than enhanced GFP, making it very easy to detect in TPLSM experiments.

By driving fluorescent proteins under the control of cell type specific promoters, it is possible to monitor the dynamics of different cell populations in their native environments (Figs. 1–3; Supplementary Movies 1–3). In the field of immunology, many transgenic and knock-in reporter mice have been generated that facilitate visualization of both innate and adaptive immune cells by TPLSM (see review by Hickman et al., 2009). The primary advantage of studying fluorescent protein reporter mice is that cells of interest are tagged and visible as long as they are living. Moreover, fluorescent proteins in transgenic reporter mice are not lost following cell division, but are instead continually replenished (assuming the promoter driving expression is active). Overall, fluorescent proteins have become indispensable to the TPLSM community, and the color palette is now broad enough to facilitate routine multicolor dynamic imaging studies. The downside to working with fluorescent proteins is that time/money must be invested into the construction and breeding of reporter mice. In addition, the design of multicolor experiments must be thought out carefully. Many fluorescent proteins have broad emission curves (e.g., CFP, GFP, YFP) that can give rise to spectral overlap. The interpretation of TPLSM datasets can be challenging when multiple, overlapping fluorescent proteins are used simultaneously, especially when the proteins differ significantly in their expression levels and/or brightness. Spectral unmixing algorithms are often used to deal with this problem and reassign fluorescent protein emissions to their primary channels; however, this process can result in signal loss. For example, a weak fluorescent protein like CFP can become difficult to see after spectral unmixing.

While fluorescent compounds and proteins are commonly used to label cells and structures of interest, it is also possible to illuminate naturally occurring structures during TPLSM experiments. These are often referred to as intrinsic fluorophores and include but are not limited to retinol, flavins, NADPH, collagen, microtubules, etc. (see Table 1 in Zipfel et al., 2003). Intrinsic fluorophores are sometimes viewed as problematic because they contribute to background or autofluorescence. However, it is possible to take advantage of intrinsic fluorophores to help define the anatomy of the tissue under investigation. For example, the splenic red pulp is highly autofluorescent due to the abundance of macrophages. This autofluorescence can be used to quickly identify regions of red versus white pulp in the spleen during TPLSM experiments because the white pulp emits significantly less background signal (Fig. 1; Supplementary Movie 1). Another intrinsic signal frequently collected in TPLSM experiments is referred to as second harmonic (Franken et al., 1961). Second harmonic generation (SHG) occurs when photons are combined by noncentrosymmetric structures such as collagen in tissues (Zoumi et al., 2002). During this nonlinear process, the new photons generated are twice the frequency and half the wavelength of the original photons. Thus, second harmonic signal can be predicted and collected based on the two-photon excitation wavelength (Zoumi et al., 2002). In spleen and lymph nodes, SHG is used to visualize the collagen-rich structural elements to which immune cells are sometimes connected (Fig. 1; Supplementary Movie 1). For central nervous system (CNS) imaging studies, SHG has been used to visualize skull bone (Figs. 2, 3; Supplementary Movies 2, 3) (Kim et al., 2009), meningeal stromal cells (Kang & McGavern, 2010; McGavern & Kang, 2011), as well as the reticular fiber network that forms in the brain parenchyma following parasitic infection (Wilson et al., 2009). In general, SHG can provide useful structural information about tissues, and there is no downside to collecting SHG other than the fact that one of the channels in a multicolor experiment will be occupied with the resultant signal. When the number of detectors available on a two-photon microscope is limiting, SHG can be discarded to make space for other fluorophores.

When choosing a combination of intrinsic and extrinsic fluorophores for TPLSM experiments, it is necessary to give serious consideration to how these fluorophores will be excited, detected, and spectrally unmixed (if necessary). Ideally, it is desirable to minimize the need for spectral unmixing by using fluorophores with narrow, nonoverlapping emission spectra; however, this is often not possible because many currently available tools were constructed without multicolor experimentation in mind. For example, enhanced GFP (eGFP) is routinely used as the fluorescent protein of choice to generate transgenic mice for microscopy experiments. Wild-type GFP was first cloned in 1992 (Prasher et al., 1992), and its enhanced derivative, eGFP, debuted three years later as a single amino acid mutant of the wild-type protein (Heim et al., 1995). Compared with wild-type protein, eGFP has increased photostability, brightness, and a more refined excitation peak. However, the emission spectrum for eGFP is rather broad and extends more than 50 nm. The same is true for CFP and YFP—the two most commonly used variants of GFP (Shaner et al., 2005). Their emission spectra have large shoulders that extend for more than 100 nm. Despite a considerable degree of spectral overlap, CFP, GFP, and YFP are routinely used for multicolor TPLSM experiments (Fig. 1; Supplementary Movie 1). Imaging these three colors at the same time can be achieved but is challenging and requires spectral unmixing. In some instances, the configuration of a detector array and/or relative differences in signal intensity can make simultaneous imaging impossible. When this occurs, a new palette of colors must be selected that are compatible with one another. For two-color experiments, it is preferable to pair GFP with orange or red fluorescent proteins such as mOrange, DsRed, or mCherry (Shaner et al., 2004). For experiments with more than two colors, it is best to avoid GFP altogether. Ideal color combinations include cyan (eCFP, mCerulean, mTFP), yellow (venus YFP), orange (mOrange), and/or red (mCherry) fluorescent proteins. These proteins can be collected in combination with second harmonic signal for four or five color experiments. It is also possible to pair fluorescent proteins with the aforementioned cell tracker dyes or other fluorescent probes. For example, 655-nm nontargeted quantum dots are far-red emitting and can be used with many shorter wavelength fluorescent proteins (Figs. 2, 3; Supplementary Movies 2, 3) (Kim et al., 2009). In general, the key to successful multicolor experimentation begins with the selection of a bright, harmonious color palette. As the next generation of tools and transgenic mice become available, it should become much easier to design TPLSM experiments.

Another consideration when selecting a color palette is the excitation wavelength of the two-photon laser. Because two-photon lasers are usually tuned to a single wavelength for real-time imaging experiments, it is important that all fluorophores in the sample be excited by one wavelength. For example, CFP, GFP, and YFP can all be excited using an excitation wavelength of ~915 nm. mOrange, however, is best excited at 980 nm and thus cannot be paired with CFP, GFP, or YFP if only one laser is available. An expensive (but, very useful) solution to this problem is to equip a two-photon microscope with two lasers (Zinselmeyer et al., 2009). This allows simultaneous excitation of a sample with two different wavelengths of light, thus permitting use of fluorophores with different excitation peaks. For maximum flexibility in designing TPLSM experiments, it is essential to have two lasers.

The energy at a given wavelength of two-photon light should also be considered when choosing fluorophores. Two-photon lasers typically generate light at wavelengths ranging from 700 to 1,200 nm. The power output of a Ti:sapphire laser (defined in watts) will be highest at ~800 nm and then drop off significantly as the laser is tuned to longer wavelengths (>1,000 nm). Because some fluorophores (e.g., mOrange, mCherry) are best excited at ~1,000 nm or greater, the low power output of many two-photon lasers at these wavelengths can pose a problem. Even with an ideal excitation wavelength, reduced power can result in suboptimal fluorophore excitation, and, consequently, low signal detection. This can be offset to some degree if a fluorophore is abundant in a tissue of interest. It is

also important to preserve as much light as possible through optimal laser alignment and the use of efficient light paths/detectors. Another option is to use a laser with greater power output at longer wavelengths. Optical parametric oscillation is a nonlinear technique that extends the wavelengths of standard Ti:sapphire lasers to 1,600 nm (Mojzisova & Vermot, 2011). This can improve the excitation of fluorescent proteins such as mCherry. In addition, next generation lasers are able to deliver more power at longer wavelengths. Use of these lasers can greatly facilitate the design of TPLSM experiments by increasing the number of fluorophores that can be optimally excited at the same time.

Tissue Preparations

TPLSM has rapidly advanced the field of immunology by allowing investigators to study the dynamic behaviors of immune cells in living tissues. Immune cells have been studied in a variety of different tissues including lymph node (Miller et al., 2002, 2003; Bousso & Robey, 2003; Lindquist et al., 2004; Mempel et al., 2004; Shakhar et al., 2005), spleen (Aoshi et al., 2008), thymus (Bousso et al., 2002), bone marrow (Cavanagh et al., 2005), lung (Kreisel et al., 2010; Looney et al., 2011), brain (Kim et al., 2009; Schaeffer et al., 2009; Wilson et al., 2009), spinal cord (Bartholomaeus et al., 2009), gut (Chieppa et al., 2006), liver (Egen et al., 2008), ear (Peters et al., 2008), foot pad (Zinselmeyer et al., 2008; Lin et al., 2009), skin (Gebhardt et al., 2011), eye (Abdulreda et al., 2011), etc. The methodology used to prepare a tissue for imaging is just as important as the selection of fluorophores and the configuration of the microscope itself. There are a few examples of noninvasive TPLSM imaging studies such as those conducted in the ear (Matheu et al., 2008) and footpad (Zinselmeyer et al., 2008). However, most TPLSM studies require at least some surgical preparation to expose or exteriorize the tissue. For example, the brain is usually routinely imaged by surgically thinning the skull to ~30 μm or by performing a craniotomy (i.e., removing a portion of the skull) (Xu et al., 2007; Yang et al., 2010). Both surgical procedures create a “window” for TPLSM imaging experiments; however, craniotomies induce a severe brain injury response that can give rise to confounding results (Xu et al., 2007; Yang et al., 2010). Injury responses should be considered when interpreting all TPLSM data derived from invasive surgical procedures. For brain imaging studies, skull thinning is the preferred surgical procedure because no injury response is elicited if the procedure is done correctly. This allows for imaging of cells in an undisturbed, physiological environment (Figs. 2, 3).

While it is preferred to conduct TPLSM studies intravitaly, this is not always possible, especially when an area of interest is deeper than a two-photon laser can penetrate. Imaging depths vary considerably from tissue to tissue and depend on variables such as light scattering, detector efficiency, autofluorescence, etc. Even in the best case scenario, it is challenging to exceed a tissue imaging depth of 1 mm, and for most tissues the maximum imageable depth is significantly less than 1 mm. In the spleen, for example, it is possible to image the red pulp intravitaly; however, deeper structures such as the follicles (which contain T and B cells) are very difficult to access reproducibly (Waite et al., 2011). A similar situation exists in the brain. Through a surgically thinned skull it is possible to image at a depth of up to 500 μm , which means that most of the brain is inaccessible. To access deeper structures, tissues are often extracted, sliced with a vibratome, and placed into an imaging chamber containing warm, oxygenated medium (Fig. 1) (Aoshi et al., 2008; Zinselmeyer et al., 2009). The advantage of this technique is that any portion of the tissue can be imaged under environmental control. Using splenic explants, it is possible to image immune cells in the follicles (Fig. 1). Brain slices, on the other hand, are routinely used to access structures that cannot be imaged through a skull window (Mainen et al., 1999). Lymph nodes have also been explanted and studied in imaging chambers (Miller et al., 2002, 2004; Bousso & Robey, 2003). Importantly, studies comparing immune cell motility

in intravital versus explant lymph node preparations have revealed remarkably similar results (Miller et al., 2003; Mempel et al., 2004; Shakhar et al., 2005; Zinselmeyer et al., 2005). Nevertheless, injury responses should still be considered when interpreting TPLSM data generated from tissue explants or slice cultures. It is known, for example, that after brain slicing, a marked injury response develops along the cutting surface, which is characterized by necrotic cell death and microglial activation (Brockhaus et al., 1996). Study of immune responses in this damaged area could yield confounding results. When working with tissue explants, it is also important to consider that the vasculature is not intact. This eliminates the possibility of studying immune cell migration into and out of the blood supply.

As technology develops, our ability to image deep tissue structures noninvasively will improve. Fiber optic two-photon microendoscopes have recently been developed that can be inserted into tissues for access to deeper structures (Barretto et al., 2011). This approach eliminates the need to physically remove a tissue, but surgical insertion of the microendoscope will nevertheless induce an injury response that needs to be considered. Thus, it is important that researchers continue to invest energy into the development of completely noninvasive deep tissue imaging approaches. Magnetic resonance imaging (MRI) may someday prove to be a useful alternative given that single iron-labeled cells can now be resolved in MRI images (Shapiro et al., 2006).

Microbial Immunity

Visualizing Pathogens

In the field of immunology, TPLSM was first used to study the dynamics of lymphocytes in lymph node (Miller et al., 2002) and thymus (Bousso et al., 2002). These pioneering studies provided the first insights into normal lymphocyte motility in the absence of infectious agents. TPLSM imaging studies of infectious agents followed shortly thereafter and now includes a long list of viruses (e.g., vaccinia virus, vesicular stomatitis virus, lymphocytic choriomeningitis virus), bacteria (e.g., *Mycobacterium bovis*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli*, *Streptococcus pyogenes*), and parasites (e.g., *Leishmania major*, *Toxoplasma gondii*) (Hickman et al., 2009; Coombes & Robey, 2010; McGavern & Kang, 2011). The key to studying pathogens by TPLSM is having them fluorescently tagged. This can be accomplished in two ways. One option is to label a pathogen with a fluorescent dye. For example, vesicular stomatitis virus (VSV) conjugated to Alexa Fluor 488 was used to study capture by subcapsular sinus (SCS) macrophages in a draining lymph node following subcutaneous injection (Junt et al., 2007). Similarly, *L. monocytogenes* coupled to Bodipy 630 was used to study the association of the pathogen with sinusoidal dendritic cells in the splenic red pulp just minutes after intravenous inoculation (Waite et al., 2011). By chemically conjugating a pathogen to a fluorophore, it is possible to image the earliest events following injection of that pathogen *in vivo*. The position of fluorophores in the resultant images will likely reflect the anatomical distribution and uptake of the pathogen. The downside to using this approach is that the conjugation procedure itself can alter the receptor binding site on the pathogen (e.g., when labeling a virus particle). This could in turn interfere with pathogen binding and uptake by host cells. A second disadvantage of using fluorophore-labeled pathogens is that the fluorophore will be lost during pathogen replication. Thus, this approach is typically used to study pathogen entry and capture by immune cells *in vivo*.

Another common strategy used to track pathogens *in vivo* involves tagging with fluorescent proteins. Viruses, bacteria, and parasites can be engineered to express any of the aforementioned fluorescent proteins (see Table 1 in Hickman et al., 2009 for examples). Ideally, the pathogen should be engineered so that the fluorescent protein coding sequence is

stably incorporated into its genome and can be passed on to its progeny. This approach has been used successfully for bacteria and parasites because these pathogens can usually tolerate extra genetic information. Viruses, on the other hand, are sometimes more challenging to modify. A large DNA virus like vaccinia virus (VV) will tolerate cargo genes and can be easily manipulated. In contrast, small negative stranded RNA viruses (e.g., vesicular stomatitis virus, lymphocytic choriomeningitis virus, influenza virus) are more difficult to manipulate and can be less tolerant of genetic cargo. Reverse genetic approaches are required to generate recombinants of negative stranded RNA viruses that express fluorescent proteins (Emonet et al., 2011). For example, a genetic trick was recently employed to construct a recombinant of lymphocytic choriomeningitis virus (LCMV) that expresses three RNA segments (the wild-type virus only has two segments) (Emonet et al., 2009). This extra RNA segment allowed for the generation of recombinants that carry cargo genes like eGFP (Emonet et al., 2009), TFP (Kang et al., 2011), and DsRed (Fig. 3b). When these LCMV recombinants replicate *in vivo*, fluorescent proteins are produced inside of infected cells, which can then be illuminated by TPLSM (Fig. 3b; Coombes & Robey, 2010; Supplementary Movie 3). Fluorescent protein-expressing recombinants of VSV and VV have also been constructed and studied *in vivo* using TPLSM (Hickman et al., 2008, 2011). In general, there are now many bacterial, parasitic, and viral recombinants that express different fluorescent proteins. These recombinants have greatly facilitated the study of pathogen-host interactions by TPLSM (Hickman et al., 2009; Coombes & Robey, 2010; McGavern & Kang, 2011). A disadvantage of using recombinant pathogens is that some are attenuated because of the genetic modifications. The three segment version of LCMV, for example, is highly attenuated when compared to the wild-type virus (Emonet et al., 2009). This attenuation results in a lower degree infectivity *in vivo*; nevertheless, the recombinant virus still induces a vigorous immune response that can be imaged using TPLSM. Another point to consider when generating a recombinant pathogen is that fluorescent protein expression can vary considerably based on the insertion site. Some recombinants have fluorescent reporters fused to viral proteins, whereas others produce the fluorescent protein separately. This along with the insertion site can impact how much fluorescent protein is synthesized inside the infected cell. It is, therefore, important to choose a site that maximizes fluorescent protein expression *in vivo*. Assuming that the fluorescent protein under investigation is not toxic, this approach will increase the likelihood of detection by TPLSM.

Immune Responses

TPSLM has rapidly advanced our understanding of innate and adaptive immune responses to pathogens by allowing investigators to capture movies of infections in living tissues. These movies have not only provided exciting new insights into the mechanisms by which pathogens access and spread through tissues, but they have also revealed how immune cells mount an antimicrobial response and sometimes cause severe pathology. To date, immune responses to *M. bovis* (Egen et al., 2008, 2011), *L. monocytogenes* (Aoshi et al., 2008; Waite et al., 2011), *S. typhimurium* (Chieppa et al., 2006), *E. coli* (Mansson et al., 2007), *S. pyogenes* (Lin et al., 2009), *L. major* (Peters et al., 2008), *Leishmania donovani* (Beattie et al., 2010), *T. gondii* (Chtanova et al., 2008; Wilson et al., 2009), herpes simplex virus (Vilela et al., 2008; Gebhardt et al., 2011), VSV (Junt et al., 2007; Hickman et al., 2008; Iannacone et al., 2010), VV (Hickman et al., 2008, 2011), and LCMV (Kim et al., 2009; Kang et al., 2011) have all been imaged in living lymphoid and nonlymphoid tissues using TPLSM. Several recent reviews have summarized many of these TPLSM studies (Hickman et al., 2009; Coombes & Robey, 2010; McGavern & Kang, 2011); thus, a more focused synopsis of the most recent literature will be provided below.

One of the main advantages of real-time imaging is the generation of dynamic data. Static images represent a moment in time, which leaves uncertainty about the events that preceded

or followed that moment. Dynamic imaging, on the other hand, yields four-dimensional (4D) datasets that capture a segment of time at defined intervals. To gain novel insights into antiviral immunity, we routinely use TPLSM to image innate and adaptive immune responses to LCMV—a murine as well as human pathogen. For example, Figure 1 shows the spleen of a mouse infected 7 days earlier with LCMV Armstrong. One day prior to infection, the mouse was seeded with naive CFP-tagged LCMV-specific CD8 T cells and GFP-tagged LCMV-specific CD4 T cells in order to visualize antiviral T cells. These naive cells were extracted from LCMV T cell receptor (TCR) transgenic (tg) mice containing viral glycoprotein specific CD8 or CD4 T cells (Pircher et al., 1989; Oxenius et al., 1998). Following infection, the transferred antiviral T cells expand massively in secondary lymphoid tissues such as the spleen, increasing in number from a few thousand naive precursors to millions of effector cells on day 7. Figure 1b is a static image showing the distribution of antiviral CD8 and CD4 T cells in the splenic red and white pulp following infection. This image is aesthetically pleasing but provides no information about the motility characteristics, surveillance patterns, or interactions of these cells *in vivo*. However, when this static image is set into motion, an entirely new perspective is unveiled (see Supplementary Movie 1). Movie 1 shows antiviral CD8 and CD4 T cells scanning the splenic red and white pulp in search of LCMV-infected targets. From this time lapse, it is possible to extract quantitative information such as the speed of each T cell, its displacement from a point of origin, whether it stops or not during the film, etc. All of this information refines our understanding of T cell behaviors in living tissues infected by a microbe.

While it is important to study the origination of immunity in secondary lymphoid tissues, the behaviors of immune cells operating in nonlymphoid tissues are equally important. Pathogens can infect a variety of different tissues and are a major cause of disease in humans. To uncover the mechanisms that drive these disease processes, TPLSM can be used to film pathology as it develops. For example, by imaging through a surgically thinned skull, we recently discovered that LCMV-induced meningitis (a fatal inflammatory disease that affects the lining of the brain) (Kang & McGavern, 2008, 2010) is caused in part by the massive recruitment of innate immune cells into the CNS following infection (Kim et al., 2009). Using a transgenic reporter mouse, we captured movies of GFP labeled monocytes and neutrophils (myelomonocytic cells) causing severe damage to meningeal blood vessels, which resulted in vascular leakage and the induction of seizures. We were also able to demonstrate in this model that virus-specific CD8 T cells can receive cues and divide in the meningeal space during the development of disease (Kang et al., 2011). Overall, TPLSM has vertically enhanced our understanding of fatal meningitis by allowing us to watch the development of pathogenesis in real time. Seeing myelomonocytic cells break down meningeal blood vessels at the peak of disease led us to discover that CD8 T cells participate in vascular breakdown by releasing chemoattractants that recruit these pathogenic innate immune cells (Kim et al., 2009).

In addition to promoting influx of blood-derived immune cells, pathogens normally trigger vigorous responses from tissue-resident cells. Most tissues are equipped with innate immune sentinels capable of rapidly detecting and sequestering pathogens. The brain, for example, is monitored by an elaborate myeloid cell network consisting of meningeal macrophages, perivascular macrophages, and microglia that can be visualized using CX3CR1-GFP reporter mice (Jung et al., 2000) (Figs. 2, 3; Supplementary Movies 2, 3). The lining of the brain (or meninges) also contains peripherally-derived dendritic cells (DCs) capable of professional antigen presentation (Anandasabapathy et al., 2011). These cells can be illuminated using CD11c-YFP reporter mice (Lindquist et al., 2004; Bulloch et al., 2008). Interestingly, intracerebral infection with LCMV-DsRed induces a complete transformation of brain-resident myeloid sentinels (Figs. 2, 3; Supplementary Movies 2, 3). Following infection, the cells become large, reactive, and tend to aggregate around areas of active viral

infection (Fig. 3b; Supplementary Movie 3). In addition, monocytic patrolling of meningeal blood vessels increases considerably following infection (Supplementary Movie 3). Thus, the local innate response to CNS viral infection is robust and sets the stage for the eventual arrival of blood-derived innate and adaptive immune cells.

Tissue-resident innate immune sentinels are not unique to the CNS but can be found throughout the body. Most tissues contain populations of macrophages and DCs that are anatomically poised to capture and present antigen. These cells often serve as the first line of defense against invading pathogens. Within draining lymph nodes is a population of specialized macrophages that reside in the SCS. Recent imaging studies have shown that SCS macrophages capture inactivated virus particles (VSV) within minutes of subcutaneous injection and later transfer antigen to follicular antiviral B cells, which results in their activation (Junt et al., 2007). These SCS macrophages not only capture antigen but also protect peripheral nerves within the lymph node from becoming infected (Iannacone et al., 2010). Depletion of SCS macrophages decreases type I interferon production in VSV infected lymph nodes. This allows the virus to infect peripheral nerves, travel back to the CNS, and, ultimately, cause a fatal paralytic disease. These data demonstrate that SCS macrophages are specialized sentinels that participate in antigen capture/transfer and can also protect peripheral nerves from viral infection. Despite this proficiency in antigen capture, lymph node macrophages are not equally skilled at priming antiviral CD8 T cells following viral infection. Both VSV and VV are known to infect lymph node macrophages and DCs, which initiates CD8 T cell priming in the peripheral interfollicular region (Hickman et al., 2008). T cell interactions with macrophages (promoted by DC ablation) results in suboptimal T cell activation (Hickman et al., 2011). Optimal priming of antiviral CD8 T cells following VSV and VV infection requires chemokine-directed interactions with DCs.

Innate immune cells can also participate in the sequestration of pathogens following infection. For example, TPLSM studies have revealed that parasitic infection of the liver by *M. bovis* results in the formation of an immobile cellular matrix referred to as a granuloma. The formation of this structure is seeded by Kupffer cells (liver resident macrophages) that capture *M. bovis* and promote the recruitment of other myeloid cells (both tissue resident and blood derived) (Egen et al., 2008). Interestingly, these granuloma structures are relatively poor at antigen presentation and support few interactions with surveying T cells (Egen et al., 2011). These data suggest that granulomas are designed to control (sequester) certain pathogens rather than clear them. This is supported by TPLSM studies of other parasitic infections such as *T. gondii* in the brain (Wilson et al., 2009), *L. donovani* in the liver (Beattie et al., 2010), and *L. major* in the skin (Peters et al., 2008).

Future Perspectives

TPLSM has generated exciting new insights in the field of microbial immunity. These new insights have stemmed primarily from our ability to see immune cells and infectious agents operating in living tissues in real time. With each new film comes a wealth of dynamic information about the biological process under investigation. This dynamic information has been used to address unanswered questions about microbial immunity and has, in many cases, completely reshaped our understanding of host-pathogen interactions. It is for this reason that TPLSM will remain a vital part of immunological research for years to come. For those of us equipped with two-photon microscopes, filming the immune system in action represents a new frontier ripe for discovery. With the current toolbox, it is already possible to conduct multicolor, high-resolution, high-speed 4D imaging studies in most tissues in the body. The color palette of available fluorophores ideally suited for TPLSM is growing rapidly, and new tools are continually being generated with multicolor

experimentation in mind. Lasers, optics, and detectors are also improving annually, making it easier for investigators to probe more deeply into tissues. One of the greatest challenges in the field is finding better and more efficient ways to visualize deep tissue structures noninvasively. Tissue explants represent an interim solution but are definitely not ideal due to confounding injury responses. Improvements in the production and collection of light will allow us to see deeper into tissues, but in many cases not deep enough. The development of microendoscopes that can be inserted into tissues offers a solution to the deep tissue imaging problem (Barretto et al., 2011); however, insertion of a microendoscope will induce an injury response that must be considered. It is unclear what technology will emerge as the front runner for noninvasive deep tissue imaging in the future. MRI is an exciting prospect given that single cells can now be tracked (Shapiro et al., 2006), and the means for generating the equivalent of “colors” in a MRI machine have been discovered (Zabow et al., 2008). Nevertheless, more technical developments are required before this approach could be used by the general immunology community. In the mean time, with the tools we have available today, opportunities for scientific discovery abound. Many exciting new insights into microbial immunity await illumination by TPLSM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Imaging antiviral immune responses in the spleen. Immune responses in the splenic red and white pulp can be imaged in splenic explants by two-photon microscopy. **A:** Depicts the method used to prepare a murine spleen for imaging. Following surgical removal, the spleen is glued to a plastic coverslip and then cut with a vibratome. To prevent drying, high glucose DMEM is pipetted onto the spleen while cutting (left panel). To gain access to the deeper splenic structures (e.g., white pulp) approximately 100 μm of red pulp is cut and removed from the spleen, which exposes the underlying follicles (center panel). After cutting, the portion of the spleen that remains adherent to the plastic coverslip is then placed into an imaging chamber through which warm, oxygenated high glucose DMEM medium flows. An in line heater is used to keep the medium at a constant temperature of 36.5°C. Following a 15–30 min equilibration period, 4D time-lapse TPLSM imaging can be performed using a water-dipping objective. Imaging depths of up to 200 μm beneath the splenic cutting surface

are routinely achieved using this approach. **B:** Shows an example of a four-color maximal projection of a 50- μm z-stack captured from the spleen of a C57BL/6J (B6) mouse by TPLSM. A Leica 20 $\times$ (1.0 numerical aperture, NA) water-dipping objective and a SpectraPhysics Mai Tai DeepSee laser tuned to 910 nm were used for this experiment. This stack was captured in the spleen 7 days following intravenous (i.v.) infection with LCMV. Prior to infection, mice were seeded i.v. with 2,000 naïve CFP-tagged TCR-tg CD8⁺T cells (green) (Pircher et al., 1989), and 2,000 naïve GFP-tagged TCR-tg CD4⁺T cells (red) (Oxenius et al., 1998). The CD8⁺ and CD4⁺T cells are specific to the LCMV glycoprotein, amino acids 33–41 and 67–81, respectively. Following infection, these cells divide extensively and can be easily monitored in the splenic red (RP) and white (WP) pulp. The dotted yellow line demarcates the border between the RP and WP. This line was drawn using red pulp autofluorescence as a guide. Second harmonic signal (fluorescent emission from ~410 to 458 nm) representative of collagen fibers is shown in pink and autofluorescence is depicted in blue. See Supplementary Movie 1 for the complete 4D dataset.

Figure 2.

Intravital imaging of normal brain anatomy through a thinned skull. **A:** TPLSM imaging of the undisturbed meninges and cerebral cortex can be achieved in anesthetized mice by surgically thinning the skull down to a thickness of $\sim 30\ \mu\text{m}$ (Yang et al., 2010). This results in complete preservation of the underlying structures and a minimal injury response. To prevent movement artifacts, a metal brace is attached to the skull bone with glue and then secured to a scaffold. A black rubber ring is glued to the metal brace in order to contain a pool of artificial cerebral spinal fluid (A-CSF) during the imaging experiment. A long working distance water immersion objective is then dipped into the A-CSF and areas of interest within the brain are imaged through the surgically thinned skull. The approach allows for imaging of areas from 200 to 500 μm beneath the skull surface. The exact imaging depth is dictated by how well the two-photon microscope is optimized for light detection. **B, C:** Two high power maximal projections at different depths within the same z -stack show innate immune cells positioned in the meninges (**B**) and neocortex (**C**). These projections were captured by TPLSM through the thinned skull of a naïve CX3CR1-GFP^{+/−} mouse. This reporter mouse illuminates myeloid cells (e.g., microglia, macrophages) and some dendritic cell subsets. Prior to imaging, 655-nm quantum dots were injected i.v. to visualize blood vessels (red). The worm-like cells (green, white arrows) that line the blood vessels in panel **B** are meningeal macrophages. Deeper imaging reveals the presence of ramified cells referred to as microglia (green) in the neocortex (**C**). A maximal project of a larger field of view is shown in panel **D**. This represents a projection of an 85- μm z -stack captured with a 20 $\times$ (1.0 NA) objective. The precise anatomy of brain myeloid cells (green) is more difficult to appreciate in this image because the meninges and neocortex are

displayed in a single projection. However, when smaller projections (15- μm stacks) starting at 0 μm (**E**), 20 μm (**F**), and 50 μm (**G**) beneath the skull surface are displayed, the differences in cellular morphology of the different brain resident myeloid cells (green) become more apparent. Skull bone (second harmonic signal) is shown in blue. See Supplementary Movies 2 and 3.

Figure 3.

Dynamics of innate antiviral immunity in the living brain. Panels **A** and **B** depict brain myeloid cells (green) responding to a LCMV infection at two weeks post-infection. For this experiment OT-I TCR-tg CX3CR1-GFP^{+/-} mice were infected intracerebrally with LCMV. These mice are unable to generate a LCMV-specific T cell response and thus represent a model of innate immunity to LCMV. Panels **A** and **B** show maximal projections (15-μm stacks) from a single z-stack starting at 0 μm (left), 20 μm (center), and 50 μm (right) beneath the skull surface. **A:** Meningeal macrophages (green, white arrows) that reside along meningeal blood vessels (red) become highly reactive (or, enlarged) following infection. **B:** In areas of LCMV-DsRed infection (red), microglia and macrophages (green) become very reactive and tend to aggregate around areas of infection (white arrow). Asterisks denote unlabeled blood vessels. Skull in all panels is shown in blue. See Supplementary Movies 2 and 3.