Chapter 4
Intravital Imaging of the Immune System

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Abstract Two-photon imaging of the immune system has revealed an active world of in vivo intercellular dynamics. Rather than static bystanders, immune cells have been re-cast as active participants in the local orchestration of immune responses. Their active motility and probing lead to new questions regarding how localization, motility, and cell-cell interactions contribute to immune responses. In combination with imaging, the use of fluorescent proteins and genetic approaches is also helping to define a role for endogenous receptor-ligand interactions and structural elements in supporting communication between the highly specialized hematopoietic cells that comprise the immune system. Although explanted tissue and organ systems are convenient for several applications, and can be established with minimal perturbation of cellular behavior or tissue environment many disease models and questions surrounding lymphocyte function require the development of stable imaging preparations that allow for long-term access to often difficult-to-reach tissues in the living animal. Here, we review emerging and established techniques for in vivo peripheral tissue imaging by two-photon microscopy.

Keywords Multiphoton microscopy • Lymphocyte dynamics • Antigen presentation • Lymph node

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4.1 Introduction

The advent of two-photon (2P) microscopy has revolutionized studies of cellular immunology by enabling high-resolution imaging deep into intact tissues and organs with minimal photo-damage to living cells. Two-photon excitation occurs when a fluorophore is excited by the near-simultaneous absorption of energy from two infra-red photons, each of which contributes one half of the energy required to induce fluorescence. Because there is a quadratic dependence on excitation intensity, fluorescence is constrained to the focal spot formed by the microscope objective, thereby providing an inherent 'optical sectioning' effect (Cahalan et al. 2002). In order to achieve practicable fluorescence signals, the photon density in the focal spot must be incredibly high to achieve the excited state through near-simultaneous absorption of low-energy photons, while avoiding photo-toxicity. This is achieved by use of femtosecond pulsed lasers, which concentrate photon output into brief (sub-picosecond) bursts with enormous instantaneous power interspersed by much longer dark periods (Cahalan et al. 2002). The quadratic dependence of excitation constrains fluorescence to the focal spot formed by the microscope objective, thereby providing an inherent 'optical sectioning' effect. To form an image plane, the single point of excitation is rapidly raster-scanned in the x, y dimensions by a system of computer-controlled galvanometer mirrors, creating a size-adjustable imaging plane. Images are often compiled in the axial (z) dimension as well, creating a 3-dimensional stack of images taken over time, hence 4-dimensional imaging. If we consider multiple fluorescent probes emitting at different wavelengths, often yielding real-time intercellular information in, for example examination of calcium signaling or NFAT relocation to the nucleus, in vivo imaging becomes 5 dimensional (Marangoni et al. 2013; Wei et al. 2007). One major advantage of 2P microscopy is that light scattering by particles in biological tissue decreases with increasing wavelength, and absorption by hemoglobin and other proteins is minimized at long wavelengths. Thus, the infra-red wavelengths (750–1,200 nm) used for two-photon imaging allow at least a five-fold deeper tissue penetration than confocal imaging. This is of crucial importance for imaging into intact, complex tissues such as the lymph node, where cells and cell interactions may be localized hundreds of micrometers below the tissue surface. Moreover, infrared wavelengths cause minimal phototoxicity, thereby facilitating long-term imaging because tissue damage is largely confined to the focal plane where 2-photon absorption occurs. For the same reason, photobleaching of fluorescent probes is confined to the focal plane which, during time-lapse z-stack imaging illuminates only a fraction of the imaged volume at any given time. Finally, the two-photon excitation spectra of most fluorophores and fluorescent proteins are generally broader than their single-photon excitation spectra. Therefore, a single excitation wavelength can be used to efficiently and simultaneously excite multiple probes with distinct emission wavelengths. It should be noted that the quantum efficiency of many fluorescent proteins is often enhanced or diminished by two-photon excitation relative to single photon fluorescence yields and cannot be predicted but is determined empirically (Drobizhev et al. 2011).

In summary, the inherent properties of 2-P excitation allow for greater preservation of native cell behavior in living tissue than other imaging techniques. Single cell resolution enables researchers to directly examine the dynamics of cell behavior and interactions in the immune system. Within 24 h, antigen-specific naïve cells that represent less than 0.00001 % of the lymphocytes in the body must correctly locate, communicate, proliferate, secrete cytokines and initiate a highly specific immune response. Imaging these processes in real-time has shed light on a previously unknown choreography of cell-cell interactions that support and contribute to immune system dynamics. Several articles comprehensively describe the use of 2-P microscopy for live cell immunoimaging (Zinselmeyer et al. 2009; Cahalan and Parker 2008; Matheu et al. 2011a; Dzhagalov et al. 2012). Here, we focus specifically on the preparation and implementation of intravital 2-P imaging techniques in living animals.

4.2 General Considerations for Intravital Preparation of Tissues for Two-Photon Imaging

In comparison with imaging of explanted tissues, intravital imaging is laborious and often requires prolonged (several hours) anesthesia and surgical procedures that may lead to a higher experimental failure rate. Nevertheless, intravital imaging yields imaging information under more truly physiological conditions, and enables study of processes such as lymphocyte homing, egress, and extravasation that cannot otherwise be investigated. Therefore, careful experimental design, identification of pertinent questions that can only be elucidated by live tissue imaging, and selection of the most relevant time-points are essential components to address before embarking on an intravital two-photon imaging experiment. It is often helpful to initially undertake in situ imaging of explant preparations to troubleshoot and identify critical time-points in preparation for subsequent intravital experiments. A generalized experimental workflow for development and implementation of an intravital imaging experiment is presented in Table 4.1. A list of common equipment that will be needed in most, if not every intravital imaging preparation is provided in Table 4.2.

4.3 Cell and Tissue Labeling

Immune cell imaging typically necessitates that more than one specific cell population be fluorescently labeled. This may be accomplished by staining cells with vital fluorescent dyes; by adoptively transferring cells expressing genetically encoded fluorescent proteins (FPs); or by imaging in transgenic animals expressing fluorescent proteins under a specific promoter.

Table 4.1 Work flow for the design and development of an intravital imaging protocol

1. Identify model, relevant question, and tissue (s) of interest
2. Determine how relevant cell types will be labeled and check that all PMT filters and excitation wavelengths are compatible
Endogenously expressed FPs are best for longitudinal studies of cell behavior
Many fluorophores with differing emission spectra can be excited at a single femtosecond laser wavelength. However this is not universally true. For example, eCFP and tdTomato do not have compatible excitation spectra. Dual laser excitation will eliminate this issue
3. Are cells of interest ≤400 mm deep?
Imaging into tissues to depths >300–400 mm may require additional microsurgery, or tissue slicing to expose the area of interest
4. Is the tissue of interest adjacent to vital organs or large blood supply?
Special surgical stabilization equipment such as retractors, clamps, and additional dissection practice may be necessary
5. Is the imaged tissue subject to pulse or respiratory movement (lung, heart)?
A suction window preparation may be necessary (Table 4.4)
Timing z-stack acquisition in synchrony with controlled tissue movement, as with ventilated lung makes imaging possible without the use of a suction window
6. Is the tissue easily separated from the animal body without significant damage to the organ? (e.g. ear, skin flap, spleen, pancreas)
Securing the tissue to a simple warmed platform with warmed media above or perfused over the tissue may be sufficient
If the tissue is not easily separated from the thorax of the animal consider a suction window design
7. Identify the relevant time-points for imaging by FACS assessment of tissues for cells of interest, or by in situ 2-photon experiments
Intra vital imaging and surgery are time consuming and have a high failure rate relative to in situ imaging
Determination of relevant time points should be performed in a more high-throughput, less time consuming manner
8. Design, have animal care committee approved, and practice anesthesia, surgical procedures and tissue stabilization prior to running a full experiment

The first 2P immune-imaging experiments visualized highly motile T cells that were labeled with cell-permeable amine-reactive or thiol-reactive fluorescent dyes (CFSE and CMTMR, respectively) and were then adoptively transferred into recipient animals (Miller et al. 2002). This technique has several advantages, including bright fluorescence of the cell tracker dyes that can be modulated by changing the labeling time of the cells; the availability of dyes with widely differing emission spectra enabling simultaneous imaging of multiple, discrete cell types; and the capacity to monitor cell division in vivo by sequential dilution of the dye. Beyond these dyes which are used to simply mark specific cell types, functional fluorescent probes can be used to monitor various cellular processes; for example, the use of fluorescent calcium indicator dyes to image calcium flux in vivo. Such assays provide information regarding cell activation and behavior relative to the local environment (Wei et al. 2007). Furthermore, real-time information about antigen

Table 4.2 General list of equipment and reagents for intravital 2P microscopy

Equipment/reagents	Purpose
Waterproof imaging stage	To stabilize the animal under the objective and protect imaging equipment from media leakage or spills
Anesthestic (see Table 4.3)	Necessary for surgery and for maintenance during imaging
Warming pad/stage	Anesthesia may cause a drop in animal body temperature, and exposed tissues cool easily
O-ring or sealable imaging well	To hold the organ of interest in liquid for imaging with a water dipping objective
Suture string	To maintain animal positioning, secure trachea in lung imaging
First aid or sports tape	To restrain animal limbs, or secure animal position
Fine surgical tools	Surgery as necessary
Electric shaver	Fur near surgical site should be removed prior to surgery
Temperature sensor	To measure the temperature of media and or local tissue near imaging site
70 % Ethanol	To 'wet' the incision area prior to surgery
Gauze, cotton swabs	To control bleeding during surgery and positioning of animal
Vetbond or similar	For adhering imaging apparatus or o-ring to the animal tissue
Bio-compatible glue	
Vacuum grease or Gel Seal	Additional sealing around imaging wells
RPMI1640 with or without Glucose	For perfusion over tissue or to fill an imaging well created around the imaging site. Glucose helps minimize local swelling
PBS	For hydration via ip injection in recovery studies; and to keep the surgical site moist
Ointment/antibiotics	To protect eyes/ensure recovery in longitudinal studies

presentation can be inferred from peptide-associated dye labels that become activated when cleaved, such as DQ-Ovalbumin. Thus, adoptive transfer of cells remains a convenient and powerful tool for in-vivo imaging of a variety of lymphocyte behaviors.

Despite the power and convenience of exogenous cell labeling and adoptive transfer, endogenous expression of a variety of cell-lineage specific fluorescent protein circumvents potential artifacts due to label toxicity or a high number of adoptively transferred cells. Numerous transgenic mice have been generated that express a wide range of fluorescent proteins (including eCFP, eYFP, eGFP, RFP, tdTomato). FP expression may be controlled by under specific promoters for proteins of interest (Reinhardt et al. 2009), or by a general promoter such as Rosa, β-actin, or ubiquitin. It should be noted, however, that the half-life of FPs may vary in vivo, especially when tagged to a protein of interest. Therefore, measurements of fluorescence intensity as a read-out of gene expression should be interpreted with caution. Once characterized, genetically-encoded fluorescent proteins can be employed to provide gene expression information, in addition to merely tracking cell movements. Linking of FP expression to genetic output allows analysis of subpopulations and

potential changes in behavior as a result of either gene expression or activation status in the living tissue. Finally, fluorescent proteins can be incorporated into probes for cellular signaling; for example to monitor cytosolic calcium levels, TCR signaling via Nur77 expression, or NFAT translocation (Marangoni et al. 2013; Moran et al. 2011; Wei et al. 2007).

When selecting an FP-expressing animal strain for an imaging project, a critical consideration is the expression level and brightness of the fluorescent protein. As a general guideline, if the fluorescence intensity in the cells of interest is >2.5 log units over background by flow cytometry, cells will likely bright enough to be detected by 2P microscopy. Increased laser excitation or longer scan times may be used to detect dim cells. However, controls for tissue damage and cell death should be used in such situations as laser toxicity can develop cumulatively over the course of an imaging experiment. Transgenic mice expressing free cytosolic FPs under the actin promoter are useful in cases where the cells of interest can be isolated and adoptively transferred, and have the advantage that the fluorescence signal does not become diluted by successive rounds of cell division.

Fluorescently labeled and / or blocking antibodies have been used to identify structural elements and cells in vivo, presenting a convenient way to quickly examine structures of interest or particular cell populations. Using labeled antibodies and deconvolution analysis allows for tremendous flexibility and improved information (Gerner et al. 2012), however, results of such studies that depend on the analysis of motile cells must be carefully interpreted and controlled as an antibody may significantly alter cell-cell, cell-substrate interactions or elicit aberrant signaling that may affect cell behavior. Vascular architecture may be highlighted by injection of label conjugated dextran, non-targeted quantum dots or microspheres into the blood stream.

2P microscopy of fluorescent labels can be readily combined with second-harmonic imaging of tissue substructure. Proteins with non-centrosymmetric, molecular structures, such as collagen and myosin, generate a second harmonic signal that is half the wavelength of the 2-photon excitation wavelength (Rivard et al. 2013; Gauderon et al. 2001; Zoumi et al. 2002); for example, an excitation wavelength of 900 nm yields a violet-blue signal at ~450 nm. The second-harmonic generation propagates in the direction of the excitation light, but there is typically sufficient back-scattered light to provide enough signal to be detected by a typical 2P epifluorescence imaging system.

Various methods for in vivo and in situ labeling of cells for two-photon imaging and their associated advantages and caveats are summarized in Table 4.3.

4.4 Anesthesia and General Surgical Considerations

Several combinations of anesthetics and analgesics can be used to achieve and maintain a surgical plane of anesthesia for in vivo imaging. Injectable drugs such as ketamine/xylene combinations do not require additional equipment in the

Table 4.3 Fluorescent labeling of immune cells for in vivo 2P imaging

Method	Advantages	Caveats
Exogenous label and adoptive transfer	Bright	Cells lost during staining
Purify cells of interest from naive, KO or transgenic donor. Label with cell-tracker dyes. Adoptively transfer into recipient animal either directly or after culture	Multiple colors	High purity necessary
	Rapid labeling	5–7 days maximum detection
	Any cell of interest	Multiple labels must have balanced brightness
	Cell division tracked	Transfer of excessive numbers of cells may lead to artifactual responses
	$Ca2^{+}$ imaging	
	Cell arrival can be synchronized	
	Cell death can be tracked	
Transgenic animals expressing fluorescent proteins in specific cell types, tagged to specific proteins, or as functional probes	Unperturbed endogenous population	Many FP labels are dim
Image directly without need for further labeling	Long-term studies possible	High cell density may limit single-cell resolution
	FP tagged transcription factors and cytokines yield real-time information	Some FPs rapidly photo-bleach
	Compatible with adoptively transferred dye-labeled cells	Several backcrosses may be necessary to specifically label cells of interest
	Natural second harmonic signal highlights collagen and myosin	In Cre reporter animals variable expression may alter identifiable populations
	Interactions via FRET can be monitored	
	Photo-convertible proteins can be used to study trafficking	
Dye-conjugated antibodies and other fluorescent reagents	Antibodies are available for a myriad of relevant targets	Antibodies may alter endogenous cell function and behavior
	Several choices of fluorescent label	Tissue injection or soaking of the LN must ensure antibody distribution
	Deconvolution of multiple signals is possible using commercially available software	Many conjugates are highly photo sensitive
	Lymphatics and blood vessels are easily labeled by local subcutaneous or iv injection, respectively with Q-dots or fluorescently conjugated dextrans (>70 KDa)	Antibody staining in vivo require controls to ensure specificity

microscopy room. However, monitoring and maintaining the surgical plane when using injectable anesthetics for extended period of time is challenging, particularly as 2P imaging is generally done in near-darkness due to wide-field detector (PMT) sensitivity to stray light. In contrast, inhaled anesthetics have a higher margin of safety due to administration of a constant dose that produces a stable plane of surgical anesthesia, when used with a calibrated vaporizer. Accidental animal recovery or overdose is less likely to occur. Therefore, inhalant anesthesia systems such as isofluorane are often the better choice for dedicated high-use 2-P in vivo imaging systems. Although there are several approved anesthetics and analgesics, different institutions often have different recommendations. As with any anesthesia, labored, agonal breathing is a sign of overdose with both inhalant and injectable anesthesia and the animal should be further monitored for partial recovery prior to proceeding with the experiment. Here we provide summary of widely available, commonly used and approved anesthetics for both terminal and recovery procedures is provided in Table 4.4 (Fish et al. 2008).

In general, surgical procedures should be minimally invasive, maintain circulation in the tissue of interest, and should be developed in collaboration with veterinarians and approved by animal care and use committees. Surgical sites surrounding the imaging area should be kept moist with a phosphate-buffered saline solution or hydrating gel, and the imaged tissue should be maintained at physiologic temperatures. Prior to initiating surgery, a surgical plane of anesthesia marked by unconsciousness, muscular relaxation, and lack of pain response/reflex must be established. Animal vital signs must be monitored throughout the entirety of the procedure.

Although survival surgery is possible with antibiotic treatment, local inflammation, and tissue scaring may significantly alter the results of an immunological based study of local cell behavior (Davalos and Akassoglou 2012). A semi-permanent ‘window’ into the tissue, as developed for brain imaging studies (Isshiki and Okabe 2014), is ideal as the tissue of interest is kept intact and protected from inflammation at the surgical site However, repeated survival surgery in a short period of time (a few days) may confound results by introducing additional swelling, local tissue damage, and introduction of exogenous pathogens. Other essential considerations are maintenance of blood and lymphatic flow, animal respiration under anesthesia (avoiding hypoxia), and tissue integrity. Additionally, careful dissection under a microscope to remove fat layers covering a tissue of interest while preserving blood flow will improve the label signal, imaging depth, and image quality. Intra-vital imaging also requires minimization of movement of the imaging site owing to respiration and heartbeat. Surgical sites that are the most easily exposed and imaged are those that are in the periphery and do not involve the abdominal cavity, head or neck. Organs that can be externalized (from the abdominal cavity) and stabilized such that movement from heart beat and breathing do not interfere with image stability may be achieved through a small incision in the abdominal wall. The most advanced intravital imaging involves practice, surgical skill, and the creative development of techniques to stabilize moving or fragile organs such as lung or pancreas. Many tissues have been successfully imaged intravitally and several key tissues and general procedures are summarized in Table 4.5.

Table 4.4 Anesthesia methods

Anesthetic	Dosage	Use	Route/dosing	Notes
Isoflurane or Sevoflurane	2–3 % inhalant to effect (up to 4 % for induction)	Especially useful for lung imaging during which ventilation is necessary	Inhalation	Survival surgery requires additional pain management (analgesia)
	Sevoflurane may require a higher dosage		Intubation is recommended	Precision vaporizer and gas scavenger required to avoid venting into room air
Ketamine	K: 60–100 mg/kg	Convenient when a dedicated ventilator is not available	IP	If re-dosing, use Ketamine alone – additional Xylazine will suppress respiration
Xylazine	X: 5–10 mg/kg		Monitor vital signs and re-dose as necessary	Acepromazine provides sedative effects and can be included in additional dosages
or			(e.g. every 30–45 min)	
Ketamine	K: 50–100 mg/kg			
Xylazine	X: 10–20 mg/kg			
Acepromazine	A: 2–3 mg/kg			
	Single syringe mixture			

Table 4.5 Surgical procedures for different intravital imaging preparations

Tissue	General procedure (s)	Imaging stage(s)	Selected references
Lymph node	Expose and stabilize Inguinal or popliteal LN	Inguinal: o-ring holder to isolate LN and contain media Popliteal: media well around joint	Miller et al. (2003), Liou et al. (2012), Matheu et al. (2011b), Mempel et al. (2004)
Spleen	Small incision on left side, elevate organ away from body	Suction window or moveable platform that secures organ above the animal	Ferrer et al. (2012)
Lung	Incision large enough to remove three ribs on left or right side below forearm. Ventilation system required	Suction window to stabilize local lung movement and reduce imaging artifact	Thornton et al. (2012), Kreisel et al. (2010), Looney et al. (2011)
Pancreas	Small incision on left side to expose spleen, gently pull spleen followed by pancreas away from the body	Platform with warmed media circulation or an adapted suction window as used with the lung procedure	Coppieters K et al. (2010)
Intestine	Small midline incision. Either pull from the mouse or rat body or leave secured in place with coverslip glued above. Paralytic (atropine at 1 mg/kg may be necessary)	Intestine may be pulled away from the core of the animal and secured to a small warmed imaging platform or directly imaged in the core of the animal by gluing a coverslip at the bottom of an imaging chamber to the intestine	McDole et al. (2012), Chieppa et al. (2006)
Footpad	Restrain of limb of interest with the footpad facing the microscope objective	On a warming platform mold a water-tight well or place a fitted imaging well around the leg of the animal	Sen et al. (2010), Zinselmeyer et al. (2008)
Ear	Secure ear to a warmed platform and place an o-ring over the site of interest for imaging	Simple platform that will easily accommodate a mouse or rat ear with warming capabilities	Sen et al. (2010), Peters et al. (2008)
Skin	Shave the area of interest	Using tissue-safe glue place an o-ring over the area of interest, fill the o-ring with tissue culture media or 1× PBS	Kim et al. (2010), Odoardi et al. (2007)
Spinal cord	Basic laminectomy of 3–5 vertebrae, using caution to avoid disrupting the spinal cord	Platform to support the head and tail with the mouse or rat in the prone position. Mounted small-animal tissue retractors expose the laminectomy site	Llewellyn et al. (2008), Rothstein et al. (2005)

Table 4.5 (continued)

Tissue	General procedure (s)	Imaging stage(s)	Selected references
Muscle	Restrain the animal and expose the muscle of interest	General use warming platform. Place an o-ring over the area to be imaged or build a media well around the area using inert tack	McGavern and Kang (2011), Dombeck et al. (2007), Pai et al. (2012)
Brain	Create a skin flap above the area of interest. Thin the skull	A small o-ring may be glued around the imaging area to help retain media. Or imaging may be performed directly with a few drops of media	Ishii et al. (2009), Kohler et al. (2011)
Heart	Ectopic transplant and engraftment for ease of exposure. Acquisition timed with ventilation to minimize movement	Adjustable upper and lower plates with an o-ring holder built in the middle to stabilize the imaging well	Li et al. (2013), Li et al. (2012)

4.5 Construction of In Vivo Imaging Stages

In general, an imaging stage should be constructed such that it is easily moved and compact but can be stably secured under the microscope. This may require tapping new screw holes in the microscope stage. The stage should be large enough in the x, y dimensions to support and position the animal while allowing for securing of perfusion tubes, additional stage parts, and suture string by taping. Finally, the imaging stage should be designed to prevent fluid leaks/spill-over into the microscope housing, stage, air-table or other equipment. Building a stage with raised edges is one way to protect from media leakage or spills. For some procedures, such as ear imaging, a block or platform secured to the stage is used to further support the tissue. Stage modifications may be achieved temporarily with waterproof tape, or by permanent attachment via waterproof epoxy glue or by stage-specific screws.

Several materials are suitable for the construction of an imaging stage. Plexiglass or Lexan are inexpensive, easily cut or milled, heat resistant, durable, and easy to clean. However, metal stages have the advantage of being less easily stripped by screw-in modular parts and represent a viable alternative for a high-use stage. 3D printing technology now allows for easy creation of small custom parts, including head restraints, nose cones, custom o-ring holders and vacuum-suction imaging windows. Several free 3D rendering programs designed to work with 3D printers are available online (e.g. Autodesk 123D, Blender, Wings3D). and commercial programs such as AutoCAD and Solidworks offer student and academic program versions.

A warming plate attachment is necessary to maintain constant temperature at the site of imaging. Often the animal can be warmed with a simple heating pad

placed under the whole animal. Additional local warming of the imaged tissue is necessary in the case of tissues isolated from the core of the animal, such as in skin-flap or spleen imaging, or with use of liquid on a peripheral tissue (ear and footpad). A simple warming device is easily created by attaching a resistor with thermal compound to a copper or aluminum platform. Small square resistors (~10–40 Ω) that are designed to be attached to a heat-sink will provide enough power to warm an imaging stage to 37–38 °C when attached to a variable 12 V power supply. The resistor should be placed at least an inch or two away from the imaging site to avoid getting wet or interfering with animal positioning. The warming plate may be covered with a layer of heat-resistant Vinyl tape which will allow for easy cleaning while protecting the warming device from repeated contact with tissue and liquid. A small thermocouple or thermistor probe is used to monitor the temperature of the tissue close to the imaging site, which can be regulated either by manual adjustment of the power to the heater, or by a feedback circuit.

4.6 Intravital Imaging Preparations

4.6.1 Skin

Imaging the ear pinna or footpad of the mouse or rat is a convenient way to examine the behavior of cells in the skin. Because the footpad is hairless, auto-fluorescent hair follicles do not interfere with the imaging signal. Also, the ear pinna has little hair, particularly the lateral surface, or inside of the pinna leading to the ear canal. The skin is perfused by blood vessels near the surface, and local injections are easily carried out in the footpad or pinna allowing for straightforward design of lymphocyte homing or infection/pathogen invasion studies. No surgery is necessary to visualize lymphocytes, facilitating longitudinal imaging studies in the footpad and ear pinna. Intravital imaging in the footpad and ear pinna has been used to successfully monitor several immune reactions and cell types that are native to the skin, including delayed-type hypersensitivity (DTH), parasite infections, T cell, ILC2 (type 2 innate lymphoid cells), adjuvant mobilization of dermal dendritic cells and Langerhans cells, and neutrophil behavior (Matheu et al. 2008; Peters et al. 2008; Chtanova et al. 2008; Zinselmeyer et al. 2008; Roediger et al. 2013; Kreisel et al. 2010). In comparison to the mouse, we find that deeper imaging in the ear pinna of the rat requires some surgery to remove the thicker epidermal layers, particularly under conditions of inflammation and local tissue edema. The ear and footpad are collagen-rich tissues, and the second harmonic signal from the collagen provides useful structural markers during two-photon imaging. Injection of a high molecular weight (70 kDa or higher) fluorescent dextran, or unconjugated quantum dots can be used to mark blood vessels. Here, we describe methods for preparing the footpad and ear pinna for 2P imaging.

4.6.1.1 Footpad Imaging

Once the surgical plane of anesthesia is achieved, place the animal in a prone position with the hind leg of interest extended behind the animal and over a warming plate pre-warmed and stable between 35 and 37 °C. Note that the ear and footpad are naturally slightly cooler than mouse core body temperature. Using breathable medical tape, secure the fore-paws of the animal to the stage. If a nose-cone is being used to administer isofluorane or medical oxygen, tape the nose cone and tubing securely to the imaging stage. Gently tie suture string around 2–3 toes, carefully pull the string taught, wrap it around the edge of the stage and secure it to the underside using waterproof tape. At this point the circulation to the toes of the animal should be checked by looking for swelling or significant color change. This is especially important for survival imaging and longitudinal imaging studies. The footpad and suture strings should be as flat as possible against the imaging stage. Tape the hind leg in place close to the body and the tail off to the side, away from the footpad that will be imaged. Once the animal is secure, build a well of sticky poster-tack around the footpad large enough to allow free access by the microscope objective. To ensure a water-tight seal, a layer of vacuum grease may be applied, and the leg may be shaved if fur interferes with the integrity of the well. Mold the tack around suture strings and above the foot-pad of the animal. If footpad imaging will be high-use or high-throughput a plastic well shaped to accommodate the leg of the animal may be built and placed over a thin layer of sticky-tack adhered to the animal to maintain a seal.

4.6.1.2 Ear Pinna Imaging

Ear imaging is facilitated by raising the pinna on a small plastic block or platform that is secured next to the head of the animal, and is fitted on its upper surface with a small warming plate. This will allow the animal to be placed in a natural supine or prone position rather than placing the animal laterally to lay the ear flat, which introduces more movement artifact. Place the anesthetized animal in the prone or supine position to expose the side of the ear to be imaged. The ventral side (interior) of the ear pinna is preferred for imaging due to a lower number of autofluorescent hair follicles. Secure the animal in this position using medical tape and, if necessary, gauze bundles to provide additional support. Cover the warming pad with medical tape to which the animal ear may be glued directly with cyanoacrylate (VetBond 3M™) adhesive; alternatively, the tip of the ear may be taped down to the imaging stage. For longitudinal studies the tape and animal can be removed from the imaging stage and the tape removed from the ear with gentle washing with 70 % ethanol. Once the ear is secured, either of two imaging chambers can be employed. First, for imaging of a small area an o-ring may be glued directly to the ear tissue and filled with media. If leakage near the base of the ear occurs, more glue may be applied to the outside of the o-ring and thin layer of vacuum grease

will maintain the seal. Alternatively, a moldable imaging chamber may be constructed with Sticky Tack (Poster Tack), which has the advantage of allowing for imaging closer to the animal head as the tack can be molded around the ear to create a sealed well.

The ear and footpad are collagen-rich tissues, thus a robust second harmonic signal within these tissues provides useful structural markers during 2-P imaging. Injection of a high molecular weight (70 kDa or higher) fluorescent dextran, or unconjugated quantum dots can further be used to mark blood vessels.

The use of depilatory cream or similar products is contra-indicated for imaging of immune responses, because a locally induced inflammatory response may alter endogenous cell behavior, progression or outcome of the immune response; although dendritic cell migration is reported to be unaffected careful controls for induced inflammation should be considered (Roediger et al. 2008).

4.6.2 Lymph Node Imaging

Imaging lymphocyte behavior is essential for understanding the underlying choreography and orchestration of an immune response. The development of an immune response and native behavior of lymphocytes under multiple conditions including thymic selection has been has been studied intensely within the last decade (Germain et al. 2012; Jacobelli et al. 2013; Tang et al. 2013), however, the nature of many cell-cell interactions, and underlying molecular forces that support cell-cell communication during various immune responses remain undefined.

The lymphatic system is comprised of a network of dedicated vessels that, starting from the extreme periphery, ferry lymphatic fluid, antigens, infectious particles and lymphocytes through sequentially arranged peripheral lymph nodes, eventually returning lymph to the circulation via the thoracic duct. Lymph nodes filter and collect pathogens as well as peripheral antigen bearing APCs, creating a contained local environment to facilitate rapid response upon local injury and infection (Girard et al. 2012). They are highly organized structures wherein cell-cell contacts and antigen delivery by DCs or lymph node-resident macrophages are tightly controlled by local chemokines to orchestrate the development of a pathogen relevant immune response (Phan et al. 2009; Gray et al. 2012; Germain et al. 2012).

The inguinal lymph node is a large, accessible lymph node that is easily prepared for intravital imaging. The popliteal lymph node is another popular choice for imaging, but is more challenging to achieve a stable preparation. Here we describe the original preparation of the mouse inguinal lymph node for 2-P imaging and present two designs for tissue stabilization during imaging. Additional equipment required for the imaging of peripheral lymph nodes using these methods is provided in Table 4.6.

Table 4.6 Procedure-specific equipment

Lymph node imaging	Dissecting microscope
	Necessary for the removal of fat above lymph node
	O-ring holder
	Static chamber over warming platform
	Superfusion chamber attached to stage (warmed media)
	12 mm ID rubber O-ring
	Small bundles of gauze to help position the animal
	PBS to keep surrounding tissue moist
Lung imaging	1–1.5 inch long 18–22 gauge intubation tube
	Y connector for intubation tubes
	50 mL conical tube
	Rubber stopper that fits 50 mL conical tube
	Small animal ventilator (mouse/rat)
	Vacuum pressure regulator (20–25 mmHg)
	Vacuum imaging window and stabilizing/attachment arm
	12 mm circular glass or unbreakable plastic coverslip
	Compressed room air
Spinal cord imaging	Mounted small animal retractors for laminectomy
	Laminectomy forceps
	Artificial cerebrospinal fluid (ACSF)
	Small animal holder/ restraint
	Stereotactic holder for the head and support of hind limbs may be useful to keep the spine straight during imaging

4.6.2.1 Inguinal Lymph Node Imaging Procedures

Once the animal is properly anesthetized, begin by shaving the lower abdomen and stomach with electric clippers, wet any remaining fur with 70 % ethanol, and make a mid-line incision from the lower abdomen to the base of the lungs without cutting into the peritoneum. On the side of the lymph node to be imaged, make a second cut starting from the lower abdomen midline incision along the inside of the leg down to the top of the knee forming a skin flap. Gently separate the skin-flap from the body of the animal, exposing the region of the inguinal lymph node. Ensure that the skin flap is separated enough to comfortably place the o-ring holder over the area of the lymph node without pressing against the body of the animal. Hold a rubber o-ring (12 mm ID) in a pair of forceps and apply a thin film of VetBond 3M™ to the bottom surface, wiping off superfluous glue such that only a thin layer is left. Gently place the o-ring onto the skin flap, centered over the exposed lymph node. To cure the glue from the inside of the o-ring, fill the o-ring with ~1 mL of PBS and wipe away excess cured glue that will float to surface. Excess glue can adhere to the exposed lymph node and obscure the two-photon signal. Flush the area around the outside of the o-ring with PBS to cure the glue around the outer edges of the o-ring

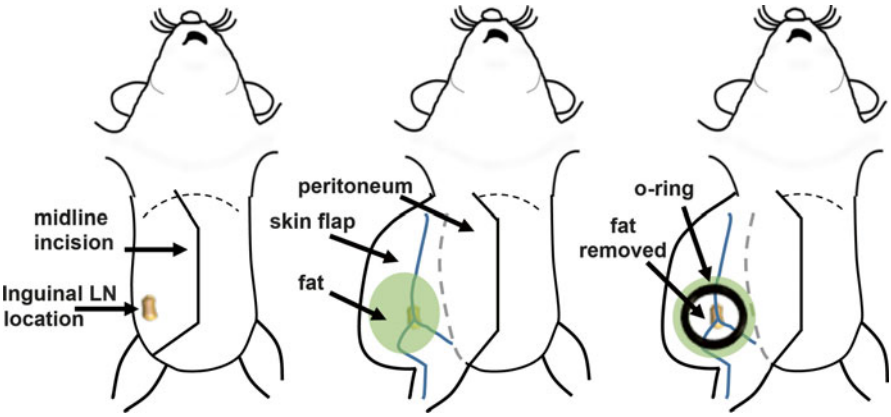

Fig. 4.1 Preparation of inguinal lymph node for in vivo 2P imaging

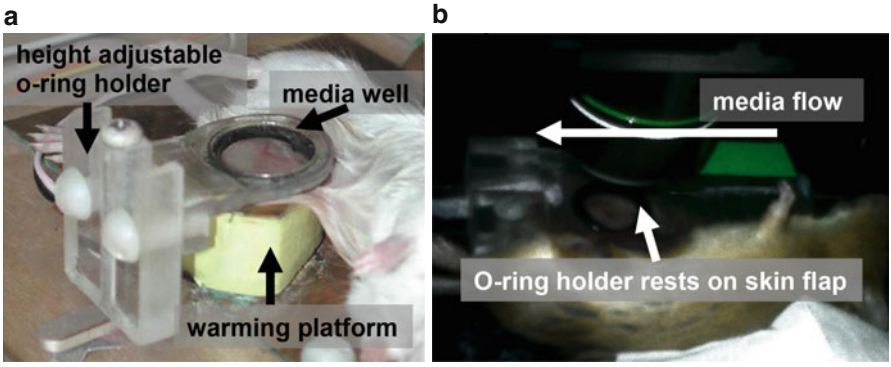

Fig. 4.2 O-ring based chambers for inguinal lymph node imaging. (**a**) Static well on warming platform (**b**) Superfusion chamber

to cure any remaining glue and ensure that the skin flap is hydrated. Soak 2 or 3 gauze strips in PBS and place them on the exposed areas of the animal to keep them moist while leaving the skin flap free. Fill the o-ring with media and place the animal under a dissecting microscope. Using fine-tipped forceps carefully remove fat around the organ while maintaining blood vessel integrity. Fat removal will be facilitated by the liquid in the o-ring which will allow it to float to the top. Replace the media in the o-ring if too much fat is present and cannot be wicked away. A schematic illustrating the o-ring preparation of the inguinal lymph node is presented in Fig. 4.1.

Figure 4.2a shows a simple design of an o-ring holder to stabilize the node and maintain physiological conditions for imaging. Once the lymph node is exposed, line the inside of the o-ring holder with a thin layer of vacuum grease, position the animal such that the o-ring is directly under the holder and gently press upwards such that the o-ring is secure in the holder. At this point the animal should be

supported by a small warming platform such that the o-ring is level with the animal and no tension is placed on the tissue. The warming platform should extend under the imaged lymph node and skin flap. Check the o-ring system for leaks and proceed with two-photon imaging.

An alternative o-ring holding system may be used conjunction with a warmed media superfusion system as seen in Fig. 4.2b, which enables improved temperature control and minimizes leakage or drying of the imaging well. A perfusion-coupled o-ring holder should be the length of the animal, fitting under the foreleg and over the leg, with a carved-out region on the underside such that the lower leg can be positioned and taped in place. In the center of the block a hole for the o-ring should be made with an inlet for media perfusion on one side and suction on the other. The underside of the o-ring holder should be thinned in the area of the skin flap such that it can be placed directly over the tissue without pressing on the skin flap and impairing circulation, while still holding the o-ring in place. Using this system, media warmed by an in-line heater may be superfused over the tissue during imaging, yielding improved temperature and imaging stability.

4.6.3 Imaging Organs of the Abdominal Cavity

When imaging organs in the abdominal cavity they must be stabilized such that breathing and heartbeat do not cause tissue movement during imaging. Several abdominal organs including lung, liver, pancreas, and intestine have been successfully imaged (McDole et al. 2012; Thornton et al. 2012; Coppieters et al. 2010; Chieppa et al. 2006; Egen et al. 2008). Here we describe a simple preparation for spleen imaging; and a suction window preparation for use in lung imaging (Thornton et al. 2012; Looney et al. 2011). The latter system is readily adapted to imaging of multiple tissues that require stabilization. We also present unpublished methods for the stable imaging of a spinal cord preparation.

4.6.3.1 Lung Imaging

Live imaging of the lung enables the behavior of lymphocytes to be visualized during a myriad of disease responses in this delicate tissue. However imaging depth is typically limited to less than 100 μm by the difference in refractive index between air in the alveoli and tissue which is closer to that of water. To image deep in the lung, a slice preparation is a useful way to examine cellular behavior (Thornton et al. 2012; Matheu et al. 2013). Additional equipment and materials for lung imaging can be found in Table 4.6.

A diagram of the ventilation system and intubation is provided in Fig. 4.3a. Imaging the inflated lung requires a custom vacuum window as shown in Fig. 4.3b, stable intubation, a mechanical ventilation system (e.g. Harvard Apparatus or Kent Scientific), an isofluorane vaporizer, and a positive end-expiratory pressure

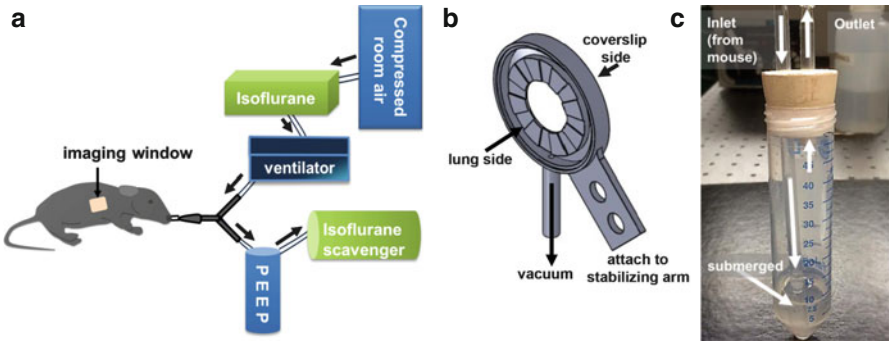

Fig. 4.3 Vacuum window system for in vivo lung imaging. (**a**) Ventilation system and mouse intubation. (**b**) Custom vacuum window. (**c**) Custom-made positive end-expiratory pressure ventilation system (*PEEP*). (Images courtesy of the Biological Imaging development Center, UCSF)

ventilation system (PEEP) that may be constructed in-house. The imaging window system described here was developed and designed in the Krummel laboratory at UCSF. For anesthesia, isofluorane-perfused room air is best used due to acid build-up associated with pure oxygen usage over an extended period of time. A PEEP is easily created by submerging the outflow from the intubated animal under ~ 3 cm of water in the bottom of a stoppered conical tube (Fig. 4.3c). Outflow from the conical tube should be collected via a second tube (not submerged) and fed into an isofluorane scavenging system.

Full-sized mice (20–25 g) are best used for lung imaging to improve the success rate of surgery. First set up the vacuum window system with a small round coverslip secured to the flat part of the window with a thin line of vacuum grease, taking care not to have any grease enter the grooves that will allow for suction. The conical side of the window should be facing down and will make contact with the lung itself. The window arm should be secured using a compact right-angle clamp holder (Bosshead style) which will allow it to be secured to a vertical support rod screwed into the microscope stage. This will allow for easy positioning in the x, y and z-direction relative to the lung. The animal may be anesthetized with either injectable anesthetic or isofluorane. Shave the area of the animal where lung imaging will be performed (typically left or right side, just under the foreleg), also shave the underside of the throat for the intubation surgery. If isofluorane is being used, intubation surgery must be performed quickly (within 1 min) such that the animal does not regain consciousness. Extensive practice of this procedure is recommended prior to live animal use. The general procedure for intubation requires that an incision of 2–3 cm is made from just even with the jaw, down to the collar bone of the animal. Next, gently pull aside the skin, then the muscle covering the trachea to expose at least ½ cm of the trachea. Thread a suture string under the exposed trachea, partially transect the trachea above the string and immediately intubate the animal. Tie the suture string around the trachea and intubation tube making an air-tight seal.

Once the tracheal intubation is complete and the animal is stability ventilated, turn and position the animal on its side exposing the shaved area for surgery. Folded

gauze is placed under the animal to support the thorax and improve lung exposure. Tape the animal in place on the heated stage. Make a small incision on the side of the animal under the forearm or on the chest and cut away the skin above at least three ribs. Carefully cut three ribs by gently cutting them at either side of the incision and removing them, cauterizing as necessary. This will expose the outside of the lung. The exposed lung should be inflated and moving in time with the respirator. Move the animal and stage under the microscope and lower the vacuum window with mounted coverslip onto the exposed lung tissue. Once the vacuum pressure is stable (between 20 and 25 mmHg; 0.02–0.03 bar) and the lung is secure against the coverslip, place a drop or two of media on top of the coverslip, lower the microscope objective and focus on the tissue. During imaging, monitor the vacuum pressure and replace evaporated media as necessary to maintain the image.

4.6.3.2 Spinal Cord Imaging

Spinal cord imaging methods have been reported by several groups, utilizing similar preparations to expose the dorsal side of the spinal cord for imaging of axons and infiltrating lymphocytes (Johannssen and Helmchen 2013; Davalos et al. 2008; Davalos and Akassoglou 2012; Steffens et al. 2012). It should be noted that the ventral side of the spinal cord is not readily accessible to intravital imaging, and the high lipid content of the spinal cord precludes imaging through the thickness of the cord. Therefore, if the cells of interest are located on the ventral side, an ex-vivo preparation may yield better imaging results.

For intravital imaging, place the anesthetized animal in a prone position on a warmed imaging stage. To keep the back as straight as possible, it is necessary to support the tail and head of the animal. Support can be achieved with a folded and secured piece of gauze or fixed platform that will allow for the nose cone to remain on the animal. Shave the back of the animal around the site where surgery will be performed and clean the surgical site with ethanol to remove any remaining stray fur. Make a 1½–2 cm longitudinal incision in the skin, centered above the imaging site. Carefully retract the skin and the underlying muscle by pulling them to the side and placing small retractor clamps or small spinal cord clamps on either side to keep the spine exposed. Choose an area in the center where the lamina will be removed. Carefully cut the lamina free by lifting the spine with forceps and sliding small, very sharp surgical scissors under the lamina and cutting the bone on both sides. The lamina should be removed in one piece with no further tugging or pulling on the spinal cord. Any bleeding may be controlled by using a small vessel cauterizer, or by gently placing pressure on the area with a small piece of gauze, kimwipe, or gelfoam. Rinse the surgical site with warmed artificial cerebro-spinal fluid (ACSF) and fill the well created by the skin and muscle retractors with ACSF. If a larger imaging site is desired remove 1–2 more lamina exposing a length of spinal cord that can be scanned for cells of interest. Add ACSF to the incision site and proceed with imaging. If additional depth around the imaging site is needed Gelseal™ (Vascutek) or similar biologically inert gel may be used to form a deeper well. Because it is

difficult to directly warm the spinal cord, the fluid well above the cord may be superfused with warmed media, or the animal may be placed in a warming chamber kept at 37 °C. Animals may be recovered from the laminectomy and re-imaged several weeks or days later, however due to the open-air nature of 2-photon imaging recovery will necessitate antibiotics, analgesics, and further animal monitoring.

Additional equipment and materials for spinal cord imaging can be found in Table 4.6.

4.7 Summary

For in vivo examination of cell behavior, video-rate two-photon imaging is unparalleled by any other imaging technology. Two-photon imaging allows cells to be visualized deep inside living tissues for long periods of time with minimal photo-toxicity. Although in situ preparations are often more convenient and can yield more data per animal, intravital imaging remains a necessary technique for any imaging that requires intact blood and lymphatic flow. With continued development of multi-color reporters and novel analysis methods, two photon imaging both in situ and in vivo will contribute novel insight to our understanding of immune response orchestration.

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