Video Article

# Dissection and 2-Photon Imaging of Peripheral Lymph Nodes in Mice

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## Abstract

Two-photon imaging has revealed an elegant choreography of motility and cellular interactions within the lymph node under basal conditions and at the initiation of an immune response [1]. Here, we present methods for adoptive transfer of labeled T cells, isolation of lymph nodes, and imaging motility of CD4+ T cells in the explanted lymph node as first described in 2002 [2]. Two-photon imaging of immune cells requires that the cells are fluorescently labeled, either by staining with a cell tracker dye or by expressing a fluorescent protein. We demonstrate the adoptive transfer procedure of injecting cells derived from donor mice into the tail vein of a recipient animal, where they home to lymphoid organs within approximately 15-30 min. We illustrate the isolation of a lymph node and describe methods to ensure proper mounting of the excised lymph node. Other considerations such as proper oxygenation of perfused media, temperature, and laser power are discussed. Finally, we present 3D video images of naive CD4+ T cells exhibiting steady state motility at 37°C.

## Video Link

The video component of this article can be found at https://www.jove.com/video/265/

## Protocol

## 1. Adoptive transfer of cells:

Tail vein or intraocular injection are both suitable methods for the introduction of cell tracker-labeled or fluorescent protein-expressing lymphocytes. In this video. we demonstrate adoptive transfer of cells by tail vein injection.

**a. Materials**

CD4+ T cells are labeled with 1.4 μM CFSE for 30 minutes at 37ºC, washed twice, and re-suspended in 100 μL of RPMI-1640. Cells are loaded into a sterile 28 gauge insulin syringe for injection. The number of cells used depends on the experiment being done. Typically, 5 million T cells are enough for simple T cell visualization. A rodent restrainer for the mouse is needed to prevent injury to the animal and to yourself.

**b. Securing the animal**

The mouse is secured for adoptive transfer by slowly backing the animal into the rodent restrainer (see video) and closing the open end of the tube by gently depressing the plunger. Do not let go of the mouse tail during this process, as some smaller animals can turn around inside the restrainer. This method prevents the animal from injuring itself and from moving during the injection. When setting up the rodent restrainer, do not push the plunger in further than is necessary to keep the animal from jumping forward. Also, do not push the plunger against the animal, and do not leave the animal in the restrainer for an extended period of time.

**c. Visualizing the tail vein**

Gently pull the tail towards you so that it is lightly wrapped over your index finger and held against the inside of the finger by your thumb. Identify the tail veins located on either side of the upright tail. Visualization is facilitated by warming the tail with warm water, then wiping down with ethanol.

**d. Injection of cells**

When the vein has been located, insert the syringe bevel-side up at a shallow angle parallel to the vein. Once in the vein, gently depress the plunger; if you are in the vein there should be NO resistance to the injection. If there is ANY resistance, remove the syringe and try again in a different location.

If you are in the vein, slowly depress the plunger until the injection is complete. The tail should not become distended during the injection. If it does, this would mean that the needle is not in the vein. It is possible to "lose" the vein during the injection. If this happens, remove the needle and try your injection in a different spot. If you need to attempt more than one injection, it is best to move the tail up towards the body of the mouse from the original injection site. Plan ahead for this possibility by starting distally on the tail to give yourself room for another attempt at the adoptive transfer.

**e. Post-injection considerations**

After the injection is completed, a small back-flow of blood from the vein will occur. Gently press the injection site with a clean-wipe until the bleeding stops. Remove the plunger and let the animal walk forward, out of the restrainer, while gently holding onto the tail.

Always check with your animal care committee and animal use protocols to ensure you are in compliance. It is important to practice this technique several times before trying the real experiment.

## 2. Lymph Node Isolation

Isolation of lymph nodes for two photon imaging requires careful removal of the correct lymph node from the surrounding tissue.

**a. Selection and location of peripheral lymph nodes**

Selection of lymph nodes for imaging will depend on the experimental goals. Be aware of local infection or injection-draining lymph nodes, and select the appropriate node for imaging. The article by Van den Broeck et al., describes the location and morphology of murine lymph nodes in detail[3]. In this video, we demonstrate the excision of six large peripheral lymph nodes which are suitable for cell isolation or imaging.

**b. Lymph node removal:**

Keep in mind the integrity of the tissue while removing lymph nodes. Be careful not to rupture the capsule or in any way damage the lymph node structure. Loss of lymph node structural integrity will compromise the experiment. If necessary, remove fat from the lymph node surface with fine dissecting (#5 Dumont) forceps under a dissecting microscope. Lymph nodes should not have any remaining fat on the surface of the node, as this will obscure imaging by scattering the light.

Proper excision of lymph nodes is also an important technique to practice before the first real experiment.

## 3. Lymph Node Imaging Setup and Considerations

The lymph node should be secured to the imaging stage, which in this case consists of a recessed chamber. Warmed, oxygen-perfused media is pumped into the chamber from one side using a peristaltic pump and inline heater, and it exits via a downward-graded channel into a collecting chamber with a vacuum tube to a waste collection flask. In our system, the lymph node is submerged in 37°C media and super-perfused with medical grade carbogen (5% $CO_2$, 95% $O_2$). The temperature of the perfusion media should be recorded as close to the lymph node as is possible. Your particular system may require variations in the media flow rate and stage design to accommodate the microscope stage and objective.

**a. Lymph node imaging setup:**

As shown in the video, we secure the lymph node by first attaching it, medullary-side down (to image T or B cell zones using an upright microscope) to a plastic coverslip, cut to the appropriate size. It is important to use a non-toxic tissue glue (we use VetBond™ from 3M Corporation).

The coverslip is secured in the flowing media by placing a small dab of silicone grease on the underside of the cover slip, then adhering this to the glass base of the imaging chamber.

**b. 2-Photon imaging considerations:**

Several factors can cause lymphocytes to stop moving: temperature that is too high or too low; excess laser power; compression or other damage to the lymph node; and lack of oxygen. Damage due to excessive laser power or temperatures > ~39°C is irreversible.

If cells are dim, it is typically better to increase the detector gain or the cell-labeling protocol rather than using a higher laser power to visualize cells. Optimization of labeling or expression of fluorescent proteins may be required to bring the fluorescence above the level of background autofluorescence. With reasonable gain and laser power settings, about a two-log shift of fluorescence above baseline (measured by flow cytometry) is reasonable for cell visualization.

With tunable two-photon lasers, the wavelength used for excitation should be a little less than double the single-photon excitation maximum of the fluorophore being imaged. In two-photon experiments using more than one label or fluorescent protein it may be necessary to experiment with the excitation wavelength used to find the optimal settings for both labels. Two-photon excitation can also be used to image endogenous collagen structures by taking advantage of the intrinsic blue second harmonic generation. Second harmonic generation (SHG) occurs where two photons are combined within a material and emit a single photon of twice the frequency of the original photons. In tissue, two-photon excitation occurs when the incident laser light is perpendicular to a highly ordered protein structure such as collagen.

Practical issues regarding two-photon imaging were discussed in preview reviews[4, 5].

## Discussion

In this video protocol we show the procedures for adoptive cell transfer and lymph node isolation and preparation required for imaging lymphocyte motility in a peripheral lymph node. Two-photon imaging has several advantages over confocal imaging in both explanted tissues (shown here) and in intravital preparations. Notably, use of infra-red excitation minimizes light scattering and allows for imaging ~300 micrometers beneath the capsule of the lymph node and deeper in some tissues[4]. Moreover, multi-photon excitation is restricted to the focal point of the objective, minimizing photo bleaching and tissue damage out side of the focal plane. As with any new technique, however, two-photon imaging is not a panacea and its limitations and potential pitfalls must be kept in mind[5]. Among these is the requirement that - excepting intrinsic signals such as second-harmonic generation by collagen- cells of interest must be fluorescently labeled by extrinsic probes or by expression of fluorescent proteins. The impression is thus created that these labeled cells are swimming in a black void; whereas in truth they are immersed in, and interact with a complex environment of structural elements and myriad other unlabeled, invisible cells.

In the six years since introduction of two-photon imaging to immunology, this technique has allowed researchers to address long-standing questions by directly visualizing in intact living tissues processes including cell-cell interactions, cell motility and localization, cellular signaling pathways and cytotoxic cell killing events. The field has rapidly evolved beyond initial phenomenological descriptions, and quantitative analyses together with computer modeling and simulation are now beginning to make sense of how the apparently chaotic cellular motions and interactions within lymph nodes lead to an efficient immune response. This progress is comprehensively reviewed in a recent publication[1], which lists over 100 papers describing the application of two-photon microscopy for immunoimaging.

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