Chapter 2

Analysis of Thymocyte Migration, Cellular Interactions, and Activation by Multiphoton Fluorescence Microscopy of Live Thymic Slices

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Abstract

Thymocytes migrate through discrete compartments within the thymus, engaging in cellular interactions essential for their differentiation into functional and self-tolerant T cells. Thus, understanding the temporal and spatial behavior of thymocytes within an intact thymic microenvironment is critical for elucidating processes governing T cell development. Towards this end, we describe methods for preparing thymic explant slices, in which the migration of thymocytes through three-dimensional space can be probed using time-lapse, multiphoton fluorescence microscopy. Thymocytes, enriched for developmental subsets of interest, are labeled with cytoplasmic fluorescent dyes, and seeded onto live thymic slices that express an endogenous, stromal cell-specific fluorescent reporter. In response to chemotactic cues produced by thymic stromal cells, the labeled thymocytes migrate within thymic microenvironments and engage in cellular interactions that recapitulate a physiological system, which can be readily imaged. Here we describe specimen preparation that maintains the integrity of thymic structures. We also describe imaging protocols for acquiring multiple fluorochrome channels to enable detection of thymocyte:stromal cell interactions and quantification of relative intracellular calcium levels to monitor T cell receptor activation. Parameters for quantifying motility and interaction behaviors during data analysis are also briefly described. The thymic slice is a versatile tool for probing live cell behaviors and developing novel hypotheses not readily apparent by static experimental methods.

Key words Multiphoton fluorescence microscopy, Thymocyte, Thymus, Migration, Cell–cell interaction, Calcium flux, TCR activation

1 Introduction

Sequential stages of T cell maturation are coordinated with ordered migration through discrete compartments of the thymus [1, 2]. The diverse stromal cells within the thymus orchestrate the localization and timing of thymocyte movement through different thymic regions in order to provide signals for thymocyte survival, proliferation, and differentiation [3]. Thymocytes respond to stromal-derived migratory cues through the regulated expression of chemokine receptors and integrins, and in turn engage stromal

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cells in crosstalk that modulates stromal cell differentiation [4]. Thus, describing the migratory behavior of thymocytes is essential for understanding T cell development. Though the importance of thymocyte migration in distinct thymic microenvironments is well appreciated, the molecular cues that drive thymocyte localization and interactions with stromal cells are not completely understood. Insights into these mechanisms are often inaccessible using standard immunological techniques, which either offer snapshots of cellular processes or fail to recapitulate processes that occur only in organized, three-dimensional tissues. Mechanisms governing thymocyte localization or interactions with stromal cells that promote thymocyte differentiation or selection can be queried by quantifying parameters of thymocyte migration as it occurs in situ.

Two-photon/multiphoton microscopy enables the visualization of fluorescent structures within tissues, due to a nonlinear excitation phenomenon that results in decreased light scattering and improved optical sectioning [5]. Thus, multiphoton microscopy is a valuable tool for observing fluorescently labeled live cells as they migrate within intact tissue. However, imaging within the thymus poses technical challenges: because the thymus is located next to the heart, intravital imaging is subject to motion artifacts [6]. In addition, central medullary regions are buried in the center of intact thymic lobes, where they are often at the detection limit of multiphoton resolution [7]. In order to circumvent these issues, techniques have been developed to generate live slices of thymus that are maintained under physiological conditions [6]. By the introduction of both exogenous and genetically encoded fluorescent markers into thymocyte and stromal cells in the system, the migration and behavior of thymocytes can be visualized by live cell imaging within the intact thymic microenvironment [7, 8]. Although thymic slices are tissue explants, thymocytes within slices remain responsive to chemotactic cues released by thymic stroma, localize to the thymic microenvironments appropriate for distinct stages of differentiation [8], and retain motility comparable to thymocytes migrating in intact thymic lobes explants [7]. Chemotactic responsiveness and thymocyte migration occur in both human and mouse thymic slices [9]. Positive and negative selection of thymocytes, which are critical checkpoints governing development of functional, self-tolerant T cells, are also supported on thymic slices [6, 7, 10]. Thus, the thymic slice has proven to be invaluable in the application of multiphoton microscopy to study the motility, localization, and stromal interactions of thymocyte subsets.

Positive and negative selection are driven by activation of T cell receptors (TCRs) on thymocytes, which is induced by ligation of self-peptide:major histocompatibility complex molecules on thymic antigen presenting cells (APCs). The concentration of intracellular calcium, a secondary messenger downstream of TCR activation, has been shown to rapidly increase and fall within

seconds of TCR activation [11] and can therefore serve as a proxy for TCR signaling. With the availability of many calcium-sensitive fluorescent indicators, live cell tracking of intracellular calcium has become an important tool in studying T cell activation [11, 12]. Cell-permeant dyes, such as Indo1 and Fluo-3/4/5, have altered fluorescence emission properties upon binding intracellular calcium [13, 14]. Chameleon is a genetically encoded fluorescent protein, whose fluorescence depends on calcium-dependent binding [15]. Among these indicators, leak-resistant Indo1, also known as Indo-PE3, has been used in imaging thymic slices, due to ease of loading and relatively bright intensity [6]. In addition, Indo1 emission is ratiometric, with intensity at shorter wavelengths increasing at higher calcium concentrations, while intensity at longer wavelengths decreases concomitantly, allowing it to serve as its own internal fluorescence control. Previous studies that quantified intracellular calcium in thymocytes undergoing selection demonstrated that thymocytes exhibit reduced motility upon calcium flux [6] and form aggregates with elevated calcium levels [16]. Further, the strength and frequency of calcium signaling have been used to distinguish positive and negative selection events [17]. Transient calcium fluxes, induced by relatively low avidity ligands, are integrated during positive selection [18], while negative selection is marked by sustained calcium elevation induced by high avidity ligands [19]. Thus, multiphoton imaging of intracellular calcium has advanced the study of thymocyte activation as it occurs in real time within the thymus.

This chapter serves as a detailed protocol of thymic slice generation. Here we describe how isolated thymocyte subsets can be labeled and introduced into thymic slices for the purpose of multiphoton imaging. Multiphoton imaging of thymic slices can yield novel insights into the molecular and cellular mechanisms that govern real-time behavior of thymocyte migration, thymocyte:stromal cell interactions, and TCR activation.

2 Materials

2.1 Preparation of Thymocytes

1. Mice: Select strain based on thymocyte subset or molecule of interest. For example, *Rag2*$^{-/-}$ mice can be used to image thymocytes blocked at an early maturation stage [20]; chemokine receptor-deficient strains such as *Ccr7*$^{-/-}$ can be used to study the role of CCR7 on thymocyte motility [21]; and TCR transgenics, such as OT-I mice [22], can be used to study selection of thymocytes with a defined TCR specificity.
2. Rodent euthanasia: CO_2 gas and chamber (VetEquip, Livermore, CA).
3. Spray bottle of 10% ethanol in water.
4. Paper towels.

5. Dissection instruments: surgical scissors (Roboz RS-5910), fine spring scissors (Roboz RS-5668), angled surgical scissors (Roboz RS-5918), forceps (Roboz 5), angled forceps (Roboz 5/45), curved forceps (Roboz RS-5135).
6. 40 μm nylon mesh cell strainer.
7. BrightLine hemacytometer.
8. Trypan blue.
9. Brightfield tissue culture microscope.
10. Fluorescent probes: CellTracker dyes (Molecular Probes) are cytoplasmic loading dyes available in a wide range of colors; Indo1AM is a cell-permeant, leak-resistant ratiometric calcium indicator dye.
11. Rat anti-mouse antibodies for depletion of hematopoietic lineages, e.g., anti-B220 (clone RA3.3A1/6.1), anti-Ter119 (BE0183), anti-Gr1 (RB6-8C5), anti-CD11b (M1/70); or thymocyte subsets, e.g., anti-CD3 (17A2), anti-CD4 (GK1.5), anti-CD8 (53.6.72), anti-CD25 (PC-61.5.3).
12. DNYNAL™ Dynabeads sheep anti-rat (Invitrogen) magnetic beads.
13. Dyna-Mag 15 magnet.
14. DRPMI medium: powdered RPMI 1640 medium deficient in phenol red, sodium bicarbonate, and l-glutamine, supplemented with 0.2 g/L sodium bicarbonate and 20 mM HEPES.
15. DRPMI with 10% bovine calf serum (BCS).
16. Phosphate buffered saline (PBS).
17. PBS with 2% BCS.
18. Complete RPMI medium: RPMI 1640 medium, supplemented with 2 mM L-glutamine, 50 U/mL penicillin, 50 mg/mL streptomycin, and 10% (v/v) fetal bovine serum (FBS).

2.2 Generation of Thymic Slices

1. Mice: 3–4 weeks of age; select strain based on thymic microenvironment and endogenous fluorescent reporters of interest. For example, in RIP-mOVA mice, medullary APCs express ovalbumin as a TRA [23], and can thus be used to study negative selection of OT-I TCR transgenic thymocytes; *Aire*^{EGFP} knockin mice express green fluorescent protein (GFP) in AIRE+ medullary thymic epithelial cells [24]; MaFIA express GFP in *Csf1r*-expressing macrophages [25]; and CD11c-EYFP express yellow fluorescent protein in CD11c+ dendritic cells [26].
2. Rodent anesthesia: Isoflurane (Southmedic, Ontario, CA) and vaporizer chamber (VetEquip).
3. Rodent guillotine (World Precision Instruments, Sarasota, FL, cat. no. DCAP or similar).
4. 60 × 15 mm tissue culture dish.

5. BioLite 35 mm tissue culture dish.
6. PTFE-coated stainless steel double-edged razor blade.
7. 5 mL volume plastic specimen cups.
8. Superglue.
9. Tapered end micro spatula, spoon spatula.
10. 4% (w/v) low melting point agarose in PBS.
11. T-type thermocouple with thin flexible probe.
12. VT-1000 vibratome.

2.3 Seeding of Labeled Thymocytes into Thymic Slices

1. Millicell 0.4 μm, 30 mm diameter cell culture membrane inserts (EMD Millipore).
2. Silica grease.

2.4 Acquisition by Multiphoton Microscopy

1. Upright fluorescence microscope with detector array, fluorescence emission filter sets, water immersion objective lens, titanium:sapphire laser for multiphoton excitation, and laser scanning system. Our setup employs the PrairieView Ultima IV (Bruker, Billerica, MA), 20× NA 0.95 Plan Fluor water immersion objective (Olympus, Tokyo, Japan), photomultiplier tube (PMT) detectors, 400/50, 480/40, 535/50, and 607/45 bandpass emission filters (Chroma Technology, Bellows Falls, VT), and two MaiTai HP lasers (SpectralPhysics, Santa Clara, CA).
2. Image acquisition software: microscope vendor specific, our setup employs PrairieView (Bruker).
3. Heated stage chamber RC-26GLP (Warner Instruments, Hamden, CT).
4. Inline perfusion heater SH-27B (Warner Instruments).
5. Nylon specimen harp SHD-26GH/2 (Warner Instruments).
6. Perfusion 300 mL IV set and flow regulator (Wolf Medical Supply, Sunrise, FL, cat. no. RF5600).
7. Bubbling stone and PVC tubing (Warner Instruments).
8. 95% oxygen with 5% carbon dioxide.
9. Imaging perfusion medium: powdered RPMI 1640 medium deficient in phenol red, sodium bicarbonate, and l-glutamine, supplemented with 2 g/L sodium bicarbonate, and 5 mM HEPES. Adjust pH to 7.4, then add 0.85 mM calcium chloride for a final calcium concentration of 1.25 mM.

2.5 Image Analysis of Slices

1. Imaging data analysis software: Analysis programs vary in accessibility of the user interface and price. For our analysis we employ Fiji/ImageJ (National Institutes of Health, Bethesda, MD), Imaris version 8.2.0 (Bitplane, Concord, MA), and MATLAB version R2015a (Mathworks, Natick, MA).

3 Methods

3.1 Preparation of Thymocytes

3.1.1 Harvest Thymocytes

1. Sacrifice the mouse in CO_2 chamber and confirm euthanasia by secondary means such as cervical dislocation.
2. Secure the mouse to the dissection board in the supine position, with its abdomen exposed, by pinning down the appendages. Moisten fur with 10% ethanol to keep the fur clear of the dissection.
3. Begin dissection of the mouse by lifting the skin up at the abdomen using curved forceps, and cut the skin up the midline from abdomen to throat using scissors.
4. Make a perpendicular cut across the upper abdomen, and pull the skin away from the thin subdermal tissue layer. Sliding the scissor blades between the skin and subdermal layer will disconnect the fascia holding the tissues together.
5. Lift the subdermal tissue using curved forceps and cut just below the ribs, taking care not to cut any internal organs, then extend the cut along the base of the ribs.
6. Pierce the diaphragm with scissors, taking care not to cut the liver or lungs.
7. Cut superiorly through the ribs on both lateral sides, ending near the axilla. Using forceps lift up the front of the ribcage, exposing the heart and thymus above it.
8. Use the curved forceps to pull the thymus out of the chest cavity by pulling at its base.
9. Gently rinse the thymus by dipping once a beaker of PBS to wash off residual red blood cells.
10. Prepare a single cell suspension of thymocytes by mechanically dissociating thymic tissue through a cell strainer into 5 mL of DRPMI with 10% BCS.
11. Count the live cells under a brightfield microscope by placing a 1:1 mixture of cell suspension and Trypan Blue on a hemacytometer slide. Live cells will be round and transparent, while dead cells are stained blue. Calculate the concentration of cells per the hemacytometer manufacturer instructions.

3.1.2 Enrich the Desired Thymocyte Subset

1. Resuspend thymocytes at 2×10^8 cells/mL in PBS with 2% BCS.
2. Depending on which thymocyte subset is needed for the experiment, thymocytes are then incubated with purified rat anti-mouse antibodies specific for cell surface markers present on lineages to be depleted. For example, to enrich for CD4 single positive thymocytes, we incubate thymocytes with anti-CD8 (96 μg/mL), and antibodies against lineage markers

CD25, CD11b, Gr1, Ter119, and B220 (5 μg/mL for each); similarly, we enrich for CD8 single positive thymocytes with anti-CD4 (21 μg/mL) and the same lineage cocktail.

3. Incubate the cells in antibodies on ice for 30 min, and then wash twice by resuspending in 5 mL of PBS with 2% BCS, pelleting the cells in swinging bucket centrifuge (5 min at 163 × *g*), and discarding the supernatant.
4. Prepare sheep anti-rat magnetic beads for use at a ratio of 2:1 cells:beads by washing the appropriate number of beads twice in 1 mL of PBS with 2% BCS, separating the beads from the wash by attaching the tube to the magnetic holder and pipetting out the supernatant with a P1000 pipet. Resuspend in 1 mL of PBS with 2% BCS.
5. Resuspend cells at 7 × 10^6/mL in PBS with 2% BCS with beads.
6. Incubate at room temperature on a rocking platform for 10 min.
7. Place the tube on the magnetic holder and collect the unbound supernatant using a P1000 pipet.
8. Repeat magnetic depletion with a second round of freshly washed beads for a ratio of 4:1 cells:beads, based on the original cell count.
9. Count the depleted thymocytes (as in Subheading 3.1.1, **step k**).

3.1.3 Label Thymocytes with a Fluorescent Probe

1. Distribute 10^6–10^7 cells each into 1.5 mL tubes, such that cells from one tube will be seeded onto one slice.
2. Prepare a solution of the desired fluorescent dye by vigorously mixing the dye in DRPMI medium (*see* **Note 1**). For example, thymocytes can be imaged in the 607 nm emission channel by staining them with 2 μM CellTracker CMTPX Red in 1.5 mL DRPMI prewarmed to 37 °C; calcium imaging can be recorded in the 400 nm and 480 nm emission channels by staining with 2 μM Indo1AM in 1.5 mL prewarmed DRPMI.
3. Pellet the cells in a fixed angle centrifuge (468 × *g*), aspirate supernatant, and resuspend the thymocytes thoroughly in 1.5 mL staining solution.
4. Incubate at 37 °C in a water bath.
5. After 30 min, pellet the cells using a fixed angle centrifuge (468 × *g*), aspirate supernatant, and resuspend in 1.5 mL complete RPMI medium.
6. Incubate at 37 °C in a water bath for 30 min to allow excess dye to destain.

7. Pellet the cells using the fixed angle centrifuge, aspirate supernatant, and resuspend in 1.5 mL fresh complete RPMI medium.
8. Keep cells at 37 °C in a water bath until thymic slices are prepared.

3.2 Generation of Thymic Slices

3.2.1 Harvest the Thymus

1. Anesthetize mice using isoflurane in a vaporizer chamber, confirm sedation by toe pinch, and euthanize mice with a rodent guillotine.
2. Drain blood by blotting with paper towels, in order to obtain clean removal of the thymus.
3. Dissect the mouse (as in Subheading 3.1.1) to expose the thymus in the chest cavity.
4. Using angled scissors, excise the thymus with a single stroke at the base, while holding gently with forceps to avoid deforming the tissue.

3.2.2 Clean and Prepare Thymic Lobes

1. Gently pick up the thymus with curved forceps and rinse by dipping once in a beaker of PBS on ice.
2. Transfer the thymus into a 60 × 15 mm tissue culture or similar large dish approximately half-full with cold PBS, so that the thymus is submerged.
3. Carefully cut away any remaining connective tissue from the thymus (*see* **Note 2**).
4. Separate the two thymic lobes by first gently outlining the septum between the lobes with the blunt side of curved forceps, and then cutting the lobes apart with a razor blade.

3.2.3 Set the Lobes in Agarose

1. Microwave the 4% low melting point agarose until liquid, and then pour it into the specimen cups, one per lobe, until the cups are about half-full.
2. Use the flexible thermocouple to stir the agarose while monitoring the temperature.
3. Using the spoon spatula, transfer a lobe into the agarose once it's cooled below 38 °C but before it sets at ~35 °C.
4. Working quickly, manipulate the lobe using the tapered end spatula so that it remains upright, with the widest part of the lobe at the bottom of the specimen cup, and the convex and concave faces of the lobe oriented outwards towards the sides of the cup (Fig. 1) (*see* **Note 3**).
5. Once the agarose begins to set, transfer the cups to a small ice bath for at least 5 min so that the agarose solidifies completely.

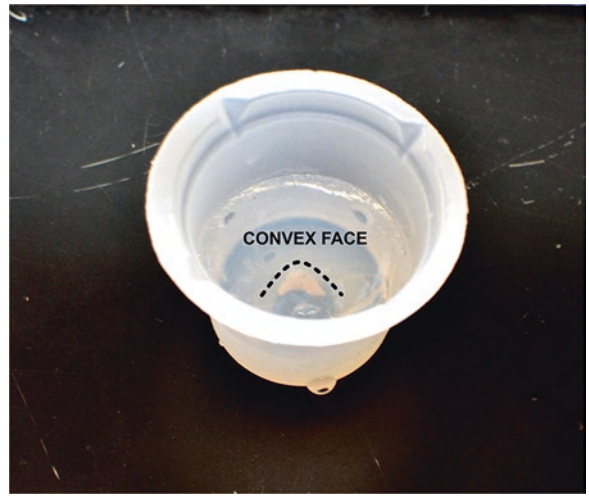

Fig. 1 Embedding the thymic lobe within low melting point agarose. Top view of specimen cup; thymic lobe is oriented vertically within the agarose so that the broader end is directed towards the bottom of the cup. Convex face of the lobe is oriented outwards, as labeled and outlined with dashed boundary

3.2.4 Prepare Agarose Tissue Blocks for Sectioning

1. Remove the set agarose block by trimming the specimen cup away with scissors and gently removing the block.
2. Use a razor blade to trim the agarose block into a cube around the lobe, leaving a few millimeters of agarose around each face of the tissue. Orient the agarose block so that the convex surface of the lobe will face the blade in the vibratome (Fig. 2a).
3. Mount the agarose onto the specimen holder of the vibratome using a thin coat of superglue, and gently press the agarose onto the holder for ~10 s to ensure good adhesion.
4. Prepare the vibratome by adding ice around the sectioning chamber and ice-cold PBS into the chamber well (Fig. 2b). Transfer and secure the specimen holder into the sectioning chamber according to the manufacturer's instructions for the vibratome.

3.2.5 Section the Agarose

1. Prepare the vibratome with a fresh razor blade and adjust the blade angle so the blade meets the agarose block at ~45 °C. Set the vibratome frequency to 70 Hz, speed to 0.20 mm/s, amplitude to 0.6 mm, and slice thickness to 400 μm.
2. Section the agarose block containing the thymic lobe continuously, collecting slices with the spoon spatula and transferring into a large dish of DRPMI with 10% BCS on ice (*see* **Note 4**).
3. Keep thymic slices in the dish on ice until ready to overlay labeled thymocytes.

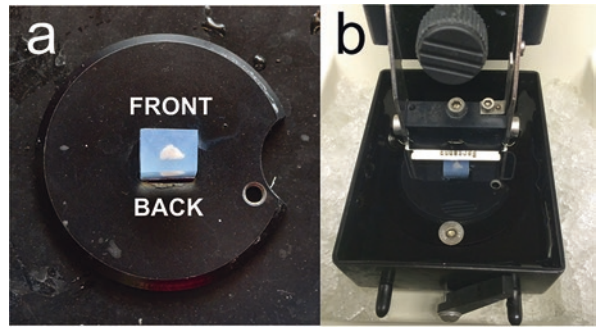

Fig. 2 Orientation of agarose block on specimen holder for vibratome sectioning. (**a**) Top view of specimen holder; agarose is trimmed with a razor so that the convex face of the lobe is aligned with the front. (**b**) Complete assembly of the vibratome setup, with the sectioning blade meeting the front of the agarose block

3.3 Seeding of Labeled Thymocytes into Thymic Slices

3.3.1 Prepare Thymic Slices for Culture

1. Place cell culture membrane inserts into small dishes with 1 mL prewarmed complete RPMI, enough to wet the membrane yet leave the culture insert at the air-liquid interface.
2. Prepare a separate dish with 2 mL of complete RPMI.
3. Pick up a thymic slice onto the spoon spatula, and wash by dipping once into the complete RPMI dish.
4. Place the thymic slice on top of the membrane at the air–liquid interface (Fig. 3).

3.3.2 Overlay the Thymocytes onto the Thymic Slices

1. Using a P1000 pipet, gently aspirate excess medium from the surface of the thymic slices without contacting the tissue.
2. Centrifuge the tubes containing dye-labeled thymocytes (fixed angle, 468 × *g*), and aspirate the supernatant so that ~30 μL remains with the cell pellet.
3. Resuspend the cells by gently triturating with a P200 pipet, and transfer the thymocytes directly onto the surface of the thymic slice. Application of a thin circle of silica grease around the border of the thymic slice can be used to further confine the thymocytes.

3.3.3 Allow Thymocytes to Migrate into Thymic Slices

Transfer the dish containing thymic slices newly overlaid with thymocytes to a 5% CO_2 incubator at 37 °C for at least 30 min before the start of imaging.

3.4 Data Acquisition by Multiphoton Microscopy

Varies with microscope platform, generally includes the following steps:

3.4.1 Setup the Imaging System

1. Warm up laser systems and tune to the excitation wavelength.
2. Mount the objective lens.
3. Assemble specimen holder onto the microscope stage.
4. Initialize data acquisition software.

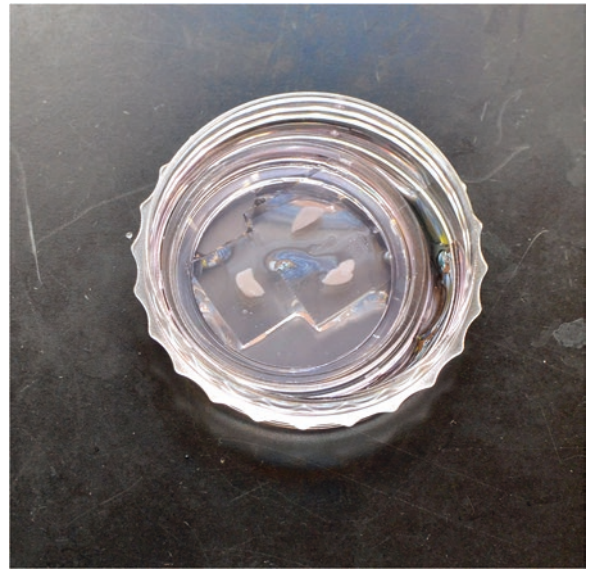

Fig. 3 Thymic slice preparation for tissue culture. Sectioned thymic slices are placed at the air-medium interface on cell culture membrane inserts. Multiple slices can be placed together for labeled thymocyte overlay. The thymic slices and seeded thymocytes are incubated at 37 °C, 5% CO_2 for at least 30 min before the start of imaging

3.4.2 Setup Stage Perfusion

1. Mount the bottle containing perfusion medium approximately 2–4 ft above the stage, and gravity fed to the stage inlet through the drip set.
2. Prime the perfusion line with a 20 cc or other large syringe, and circulate the perfusion medium to the specimen chamber with the regulator flow rate at ~100 mL/h, or ~1 drop/s. Adjust the outlet flow so that the medium can cover a thymic slice but does not overflow the chamber.
3. Connect the bubbling stone to the 95% oxygen with 5% carbon dioxide source, and feed gas into the perfusion medium so that it bubbles vigorously.
4. Heat the microscope stage and inline perfusion heater, monitoring to ensure the medium is at 37 °C.

3.4.3 Secure the Thymic Slice onto the Stage

1. Lifting the thymic slice from the petri dish using a tapered end spatula, use a P1000 pipet to gently rinse the top of the slice with warmed RPMI medium, to remove any cells that did not enter the slice.
2. Transfer the thymic slice into the warmed perfusion medium of the imaging specimen holder, so that the side of the slice on which labeled thymocytes were introduced faces the objective lens.

3. Using forceps, carefully secure the thymic slice by placing the nylon specimen harp onto the specimen holder without damaging the tissue.
4. Once in place, lower the objective lens to immerse in the medium.
5. If binocular view is available on the microscope, align the objective and roughly focus on the edge of the thymic slice, to assist in fluorescence imaging setup, taking care that the laser source is blocked before looking into the eyepiece.

3.4.4 Time Lapse Imaging of Live Cells Within a 3-D Thymic Slice Volume

1. In live view, scan the tissue to find imaging regions of interest. For example, when determining accumulation and migration of thymocytes within cortical and medullary thymic regions, the imaging field should ideally encompass a complete medullary patch and an intact cortical expanse extending from the cortical medullary junction to the capsule. Various focal depths should be explored to find a suitable imaging volume, as medullary tissue tends to be less rigid than cortical tissue and thus can have less integrity closer to the tissue surface. Alternately, if observing cell-cell contacts, the field of view should be focused onto the thymocytes and/or stromal cells at sufficiently high magnification to resolve such contacts. Other imaging parameters to be set include:
2. *Excitation intensity*—Pockels cell voltage for attenuating excitation laser intensity should be set so that the image is bright, while bearing in mind that high illumination comes at the cost of photobleaching during extended imaging times.
3. *Excitation wavelength*—For CellTracker dyes, GFP and its variants, peak excitation is found ≥800 nm. These red-shifted wavelengths are also ideal because they cause less tissue damage; however the optimal excitation wavelength must be determined based on the specs of individual lasers with respect to power emitted at each wavelength, and the combination of fluorophores to be excited by a single wavelength in a given experiment. In order to image intracellular calcium concentrations using Indo1AM, the laser must be tuned to a shorter wavelength. For our experiments observing both Indo1AM and GFP, we use simultaneous excitation by two laser sources at 730 nm and 840 nm, respectively.
4. *Focal plane interval distance (z-step)*—The top of the imaging volume should be set so that it captures thymocytes migrating within the tissue, rather than unincorporated cells floating on top of the slice. For this reason, we set the top imaging plane ≥20 μm below the cut surface of the slice. Given an average thymocyte diameter of 10 μm, we image the tissue depth at a maximum of 5 μm intervals to avoid undersampling. While

smaller step sizes can be used in order to obtain greater lateral resolution, this will increase the number of focal planes to be acquired for a given tissue volume and thus increase laser exposure and tissue damage. We generally acquire nine layers at 5 μm steps in order to observe a tissue volume depth of 40 μm. Imaging deeper than 100 μm within the tissue may be more difficult due to attenuation of fluorescence through the tissue slice, with image resolution dependent on the efficiency of the fluorophore and the density of structures labeled.

5. *Frame and/or line averaging*—The use of signal averaging will improve the signal-to-noise ratio (SNR) and provide a clearer image. However, the trade-offs include increased laser exposure resulting in photobleaching as well as increased computational load during data acquisition.
6. *PMT voltage and preamp*—Increased PMT voltage can be employed in order to minimize the amount of laser intensity that must be applied in order to obtain a bright image signal; however high gain will increase noise. For improved SNR, preamp gain and offset can be adjusted in order to cover the whole dynamic range of pixel intensities (*see* **Note 5**).
7. *Pixel dwell time*—Similar to signal averaging, increased dwell times will result in increased signal and improve image quality, with the caveat that long dwell times will increase laser exposure, inducing photobleaching and tissue damage.
8. *Time interval duration*—Duration between time points must be selected so that cellular movements are adequately sampled, while not oversampling and thus unnecessarily exposing the sample to laser illumination. For thymocyte migration within the thymic slice, we generally select intervals of 15–20 s, for total imaging durations of 15–30 min.

3.5 Image Analysis

3.5.1 Import the Image Data Files to the Analysis Software Program

The data acquisition file type should be considered since this affects their readability by the analysis program. Proprietary file types may require file conversion programs, though many dedicated image analysis programs can now read most file types and associated metadata employed by microscope vendors.

3.5.2 Calculation of Thymocyte Motility Parameters

Motility parameters enable quantitative comparisons among experimental conditions. Calculation of many parameters is automatic in the data analysis program Imaris Bitplane once thymocytes have been rendered as Spot or Surface objects. Otherwise, these parameters can be calculated manually by plotting thymocyte positions as a function of time. Useful metrics for comparing motility under different experimental conditions can include:

1. *Displacement*—the absolute distance traveled between given time points; plotting displacement as a function of the square

root of time can reveal whether the cell tends to travel in a random walk (slope ~ 1) or in a confined path, in which the plotted curve quickly reaches a plateau.

2. *Path straightness*—displacement divided by the length of the path traveled by the cell. Path straightness reveals whether the cell travels in a straight line (straightness of 1) or in a more tortuous manner (decreasing straightness value approaching 0).
3. *Velocity*—path length divided by the time duration sampled. Mean cell velocities can be impacted by such factors as cellular interactions, chemokine gradients, and activation state.

3.5.3 Quantification of Thymocyte: Stromal Cell Interactions

In order to quantify cellular interactions, the shortest distance between the surfaces of each thymocyte and stromal cell must be computed for every time point; for this purpose we have utilized the ImarisXT package of Imaris:

1. Mask the fluorescence channel marking the stromal cells (most often, stationary cells), using Surfaces.
2. Convert the datatype to 32-bit floating.
3. With the stromal cell Surfaces object active, generate a new channel using Imaris XT Distance Transformation. Specify distances outside the object. In the newly created channel, the intensity denotes distance away from the mask.
4. Once rendered as an Imaris object, each thymocyte will now have channel intensities which represent its distance to the nearest stromal cell at any given time point.
5. In order to call a time point of interaction for the thymocyte, determine a threshold distance; based on visual inspection of our data sets, we use ≤ 3 μm from the stromal cell mask as the threshold for a thymocyte:stromal cell interaction.
6. The duration of individual contact events was measured based on consecutive interaction time frames. Thus parameters such as frequency of contact, mean contact duration (dwell time), and percent of time contacting stromal cells can be quantified.

3.5.4 Calcium Imaging as a Proxy for Activation

1. We employ the ratiometric calcium indicator Indo1AM in order to visualize changes in intracellular calcium concentrations as a proxy for TCR signaling during thymocyte selection events (*see* **Note 6**). In order to obtain a relative calcium signal, the total fluorescence intensity of the cell volume from the 400 nm emission channel is divided by that of the 480 nm emission channel.
2. By plotting individual cell calcium ratios, periods of elevated or baseline intracellular calcium can be described; TCR activation

events can be determined based on relative fluxes in the calcium signal, followed by signal decay. The fluorescence ratio values that indicate peak flux and elevated intracellular calcium signals are specific to the experimental conditions, and can be determined by calibration experiments.

3. To calibrate, an ionophore, such as ionomycin, or the cognate antigen of the TCR transgenic thymocytes is introduced into the perfusion medium to induce TCR activation. The addition of the activating reagent will result in rapid calcium flux within all the thymocytes in the sample.
4. Since the time of reagent addition and thus TCR activation is known, we can obtain relative fluorescence ratio values for resting (before addition of reagent) and activated states.
5. After any calibration the perfusion line must be flushed and the specimen holder cleaned completely, as any residual ionophore/antigen will contaminate subsequent imaging.

4 Notes

1. Any excess fluorescent dye will interfere with imaging by leaving fluorescent debris on the thymic slice. It is essential that the dye is thoroughly mixed into the medium during preparation, which we accomplish by vigorous vortexing of the dye after adding to media. During the wash steps after cell staining, it is also critical that any unbound dye is removed. Towards this end, it is helpful to centrifuge tubes in a fixed angle centrifuge rotor and aspirate the non-cell debris, which distributes along the wall of the tube, away from the cell pellet in the tube.
2. Removing all connective tissue from the thymus is essential to generating high quality thymic slices for imaging. Large sections can be cut away with scissors, while smaller pieces can be carefully peeled off using angled forceps. Complete removal of connective tissue is facilitated by dissection under a stereomicroscope.
3. Along with using a completely clean thymic lobe, the manner in which the lobe is set into the agarose is critical to obtaining good thymic slices. Upon introducing the lobe into the cooling agarose, the boundaries of the lobe must completely contact the agarose, without any air pockets, to ensure that the subsequent slicing step cuts through the lobe rather than crushing the tissue. Before the agarose has set, a spatula can be used to agitate the agarose, directing it towards the lobe.
4. Several difficulties may arise during sectioning that can result in inadequate thymic slices. If connective tissue remaining on the thymic slice attaches to the blade, the blade can pull the

tissue out of the block. If there is a gap between the lobe and the agarose, the blade can crush the tissue. For these issues, slices may be salvaged by pausing the blade and trimming any uncut tissue away with fine spring scissors before resuming. It is also advised to prepare extra tissue blocks to ensure sufficient thymic slices can be obtained for imaging. In addition, large thymuses, for example from mice 6 weeks of age, may prove difficult to slice, as their large cross-sectional area may collapse after sectioning. We have found it easiest to section from mice 3 to 4 weeks of age. However, ages can be adjusted per experimental needs. While keeping the cut slices on ice will maintain tissue integrity, tissue structures look best shortly after sectioning; therefore sectioning should be timed to minimize the time the slices sit before imaging.

5. Practically speaking, there should be a balanced number of pixels that are detected with zero and maximum ($2n^{-1}$, where n is the bit-depth of the image) intensity. Thus, the greatest range and variation of intervening graytones can be acquired in the image, providing improved image resolution. Adjust PMT preamp gain and offset while observing the live image with a pixel intensity lookup table (LUT), with the goal of having sparse zero intensity pixels and very few regions of saturated pixel intensity.
6. Drawbacks to Indo1AM include a blue-shifted excitation peak wavelength compared to most fluorophores, scattering of emission fluorescence resulting in noisy signals from single cells, and changes in calcium sensitivity over time as the dye is compartmentalized, making its use practical only within the first 6 h after loading. In addition, the capacity to observe Indo1AM and GFP simultaneously has necessitated the use of two excitation lasers, which increases exposure of the tissue to phototoxicity. However, Indo1AM remains a good tool for calcium detection due to its ease of loading and brightness.

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