

Video Article

Real Time *In Vivo* Tracking of Thymocytes in the Anterior Chamber of the Eye by Laser Scanning Microscopy

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Abstract

The purpose of the method being presented is to show, for the first time, the transplant of newborn thymi into the anterior eye chamber of isogenic adult mice for *in vivo* longitudinal real-time monitoring of thymocytes' dynamics within a vascularized thymus segment. Following the transplantation, laser scanning microscopy (LSM) through the cornea allows *in vivo* noninvasive repeated imaging at cellular resolution level. Importantly, the approach adds to previous intravital T-cell maturation imaging models the possibility for continuous progenitor cell recruitment and mature T-cell egress recordings in the same animal. Additional advantages of the system are the transparency of the grafted area, permitting macroscopic rapid monitoring of the implanted tissue, and the accessibility to the implant allowing for localized in addition to systemic treatments. The main limitation being the volume of the tissue that fits in the reduced space of the eye chamber which demands for lobe trimming. Organ integrity is maximized by dissecting thymus lobes in patterns previously shown to be functional for mature T-cell production. The technique is potentially suited to interrogate a milieu of medically relevant questions related to thymus function that include autoimmunity, immunodeficiency and central tolerance; processes which remain mechanistically poorly defined. The fine dissection of mechanisms guiding thymocyte migration, differentiation and selection should lead to novel therapeutic strategies targeting developing T cells.

Video Link

The video component of this article can be found at <https://www.jove.com/video/58236/>

Introduction

Intrathymic T-cell differentiation and T-cell subpopulation selection constitute key processes for the development and maintenance of cell-mediated immunity in vertebrates¹. This process involves a complex sequence of tightly organized events including the recruitment of progenitors from bloodstream, cell proliferation and migration, differential expression of membrane proteins, and massive programmed cell death for subsets selection. The result is the release of mature T-cells reactive to an ample spectrum of foreign antigens while displaying minimized responses to self-peptides, which end-up colonizing the peripheral lymphoid organs of the individual^{2,3}. Aberrant thymocyte selection of the $\alpha\beta$ TCR repertoire leads to autoimmune disease or immune imbalance⁴ that mainly derive from defects during the processes of negative or positive precursor selection, respectively.

Directional migration of thymocytes across the thymus is intrinsic to all stages of T-cell maturation and it is envisaged as a series of simultaneous, or sequential multiple stimuli, including chemokines, adhesive, and de-adhesive extracellular matrix (ECM) protein interactions^{3,5}. The study of fixed tissues has rendered critical information regarding the patterns of expression for thymocyte migratory cues in defined thymic microenvironments^{5,6}, while *ex vivo* studies has revealed two prevalent migratory behaviors of thymocytes in two histologically distinct areas of the organ: slow stochastic movements in the cortex and fast, confined motility in the medulla^{7,8,9,10,11,12,13}. Increased migratory rates correlate with thymic positive selection¹³ and negative selection is associated with crawling behavior supporting the hypothesis that the kinetics of the journey through the thymus determines proper maturation of thymocytes. Despite their relevance, the topology of thymocyte-stromal cell interactions and the dynamics of thymocyte motility across organ microenvironments during T-cell maturation remain ill-defined.

Most *ex vivo* studies performed to date include fetal or reaggregate thymic organ cultures^{14,15}, tissue slices or intact thymic lobe explants where thymocyte movements are visualized by two-photon laser scanning microscopy (TPLSM)⁹, an intravital imaging technique with a restricted maximum working distance and imaging depth of 1 mm in accordance with the tissue examined¹⁶. In contrast to the laborious thymic organ cultures which depend on extended incubation times to form 3D-structures, both, the thymic slice technique and the intact thymic lobe approach permit controlled introduction of particular subsets of pre-labeled thymocytes into a native tissue architecture environment. However, since blood flow is absent in these models, they are clearly limited for studying the recruitment process of thymus settling progenitors (TSPs) to the thymus parenchyma or the dynamics of thymic egression of mature T-cells.

In vivo models for the study of thymic T-cell maturation physiology in mice include the grafts of fragments or entire organ lobes placed either inside the kidney capsule¹⁷ or intradermally¹⁸. Although these options showed their utility to interrogate systemic functional engraftment of the tissue, the position of thymic grafts deep within the animal or covered by layers of opaque tissue restricts their use for *in vivo* examination of implants by TPLSM.

The anterior chamber of the eye provides an easily accessible space for direct monitoring of any grafted tissue by virtue of the transparency of corneal layers. Of advantage, the base of the chamber formed by the iris is rich in blood vessels and autonomic nerve endings, enabling rapid revascularization and reinnervation of the grafts^{19,20}. Dr. Caicedo has successfully used this anatomical space for the maintenance and longitudinal study of pancreatic islets in the past²¹. Here, we show that this strategy not only constitutes a valid approach to study thymocytes' dynamics within the native organ structure, but also uniquely permits to extend the *in vivo* longitudinal recordings to the study of progenitor recruitment and mature T-cell egression steps in mouse.

Protocol

The Institutional Animal Care and Use Committee (IACUC) of the University of Miami approved all the experiments according to IACUC guidelines.

1. Isolation and Trimming of Newborn Thymi

1. Prepare all reagents and instruments by autoclaving or other methods, ensuring sterile conditions.
2. To minimize contaminations, perform all surgical procedures under a laminar flow hood.
3. Prior to euthanizing donor mice, fill a 60 mm sterile dish with sterile prechilled 1x phosphate-buffered saline (PBS, pH 7.4) and place it on ice. It will be used for rinsing and storing the excised thymi from donor mice prior to the transplantation.
4. Proceed to euthanize newborn donor mice by decapitation, in accordance with ethical guidelines.
5. Place the mouse on sterile absorbent paper towels in a dorsal upright position and spray, and later wipe, the mouse abdomen with 70% ethanol.
6. Expose the thoracic cavity by making a superficial V-shaped incision at the level of the lower abdomen and cut the skin with a pair of straight 10 cm dissecting scissors following a ventral midline to leave an opening of about 0.5-1 cm at the chest level. Fold the skin over each side of the chest to expose the thoracic cavity.
7. Make two deeper 0.5-1 cm lateral incisions through the diaphragm and ribcage with the same type of scissors to access the superior mediastinum in the anterior thoracic cavity. The thymus should appear as two pale lobes right above the heart.
8. Place a set of curved forceps underneath the thymus and pull vertically to extract the complete organ. Prevent the foldback of the ribcage with a pair of forceps. If needed, use fine forceps to carefully tear apart the connective tissue surrounding the organ without disrupting the capsule before extracting the organ.
NOTE: This step is facilitated by using a dissecting scope.
9. Submerge the isolated thymus into cold sterile 1x PBS (pH 7.4) previously displayed into a 60 mm sterile dish, and cut the connective isthmus with a scalpel to separate the thymic lobes. Remove any debris from the dish without harming the capsule. To minimize the time thymi are exposed, trim thyme lobes right before implant.
NOTE: Each isolated newborn thymus will typically render six segments of about 1 mm wide for a maximum of three host mice when receiving implants in both eyes.
10. Repeat Steps 1.4-1.9 for each donor mouse. To minimize the time thymi are exposed, isolate one thymus at a time.

2. Thymus Implantation into the Anterior Chamber of the Eye

1. Prior to the transplantation, tag and weigh each recipient mouse.
2. Use a dose between 1-2% of isoflurane vapors to anesthetize the recipient mouse. Ensure proper anesthetization by the absence of reflex following toe pinch before starting the surgical procedure.
3. Proceed with thymus segments transplant as follows:
 1. Place the mouse in a side lateral recumbent position so that one eye is facing up directly exposed to the lens of the dissecting scope.
 2. Excise one of the isolated thymic lobes lying in the cold 1x PBS (pH 7.4)-filled 60 mm dish into pieces with up to 1 mm width for implant. Follow a zig-zag pattern to ensure that each segment contains thymic cortex and medulla. Use vannas scissors for trimming according to guidelines provided in **Figure 1A**.
NOTE: Trimming of the thymus should be done right before implant to optimize tissue engraftment.
 3. Starting in the base of the cornea corresponding to the surgical area, introduce the tip of a 40 mm G18 needle to make a small incision into external cornea layers so that the dissecting scissors tip can be introduced.
 4. Make a 5-10 mm flank incision directly around the base of the cornea using vannas scissors while holding the cornea opening firmly to prevent resealing. Use forceps with flat ends to avoid tissue damage.
 5. Grasp the cut corneal epithelium and expose the opening by holding the cornea with a pair of flat-ended forceps while pushing a thymic segment through the opening. Wet the eye with sterile 1x PBS (pH 7.4) or artificial tears as needed to prevent tissue desiccation until the manipulation is complete.
 6. Press softly on the eye surface to slide the introduced tissue segment to a lateral position with respect to the pupil to preserve eye's function. Ensure that the graft lies at a location opposite to the eye opening to prevent posterior interference with imaging by hazing.
 7. With the aid of flat forceps, press firmly, for about 3-5 s, the two sides of the corneal opening against each other to promote self-sealing. The surgery does not require any stapling or sewing.
4. If the transplants are performed on both eyes, turn the mouse over the opposite lateral side to directly expose the second eye to the dissecting microscope lens and repeat Steps 2.3.2-2.3.7.
5. After the surgery is completed, return the mouse to an empty cage prewarmed with a heat lamp.

6. Make sure that each transplanted mouse regains complete mobility and consciousness before placing it into a cage shared with other animals. This usually takes place within 1 h after the surgery.
7. Upon transplant, treat the mice with pain killers as needed, and provide the mice with acetaminophen at a concentration of 1.6 mg/mL in the drinking water.
8. Repeat Steps 2.2-2.9 for each recipient mouse.
9. Monitor post-operative animal activity as well as the appearance of the implanted eyes to detect potential health complications.

3. Confocal Imaging of Implanted Thymi using 3D Single Photon Fluorescence Confocal Microscopy

1. Anesthetize the recipient mouse using a dose between 1-2% of isoflurane vapors. Ensure proper anesthetization by the absence of reflex following toe pinch before starting the imaging procedure
2. Place the mouse on a fixed stage microscope platform in a side lateral recumbent position so that one eye is facing up. A heat pad is placed on the microscope platform to ensure constant body temperature of mice during recordings.
NOTE: See **Supplementary Figure 1** for details.
3. Insert the head of the mouse into a stereotaxic headholder and adjust the knob to restrain the mouse head on a lateral side position allowing direct access of the microscope objective to the eye holding the thymus graft.
4. Place the mouse snout into a gas mask to keep the animal anesthetized throughout procedure. This allows the adjustment of isoflurane vapors as needed.
5. To facilitate the stabilization of the eye during recordings while avoiding the retraction of the eyelids, pull back the eyelids while holding the eye at the corneal margin with a pair of tweezers that have their tips covered by a polythene tube which are attached to a UST-2 solid universal joint. This arrangement permits a steady fixation of the head and eye and provides flexibility without disruption of blood flow in the eye, as previously described²².
NOTE: **Supplementary Figures 1B, C** show the complete assembly.
6. Add a few drops of sterile saline or artificial tears as the immersion liquid between the cornea and the lens, before placing the microscope objective on the mouse eye.
NOTE: Additional drops are dispensed on need throughout the procedure to keep the microscope path and prevent eye drying.
7. First use low magnification (5X) lens to locate the thymus in the microscope field. Then switch to higher resolution (10, 20 and 40X) water immersion dipping objectives with long working distance. Avoid LSM photodamage and bleaching of the thymic implant by applying minimal laser power and reduce scanning time as much as possible. This is achieved by using the resonant scanner of the microscope.
NOTE: The confocal microscope we use is equipped with resonant scanning mirrors capable of gathering images at 25 frames/s. Other brands have their own microscopes with resonant scanners.
8. Select the acquisition mode using the microscope software and start the resonant scanner mode. Then choose the XYZT imaging mode and configure the acquisition settings as follows:
 1. Turn the Argon laser on and adjust the power to 30% for fluorescence excitation.
 2. Choose an excitation laser line and set the acousto-optical beam splitter (AOBS) control for different emission wavelengths. In addition, to detect backscatter and delineate tissue structure, use reflection detection simultaneously.
NOTE: Upon selecting a set of colors for excitation (GFP and RFP), the AOBS is automatically programmed to direct these excitation lines onto the specimen and transmit the emission between them. For instance, for GFP and RFP, we use narrow reflection bands for excitation light around 488 nm and 561 nm, respectively. This leaves broad bands for the collection of emitted fluorescence photons, thus reducing the need for laser power and acquisition time. In reflection mode, the AOBS is used as a 50/50 beam splitter to image reflected light in any wavelength away from the ones used for fluorescence emission detection.
 3. Collect the emission at selected wavelength, then select a resolution of 512×512 pixels and start live imaging by pressing the **Live** button, adjusting the gain levels as needed (typical gain is around 600 V).
 4. Define the beginning and end of the z-stack by focusing on the top of the thymic implant and select **Begin**, then move to the last plane that can be focused in the implanted thymus and select **End**. Use a z-step size of 5 µm. The software will automatically calculate the number of confocal planes.
 5. Choose the time interval for acquisition of each z-stack (Typically 1.5 to 2 seconds) and select the option **Acquire until stopped** for continuous imaging.
9. Press the **Start** button to initialize.
10. Image the grafts repeatedly at different times from the same animal by repeating Steps 3.1-3.9.
NOTE: If the animal holds grafts on both eyes, recordings from either eye can be taken by repeating Steps 3.2-3.9 after switching the upright position side of the mouse head.

Representative Results

Thymus from newborn mice were isolated from *B6.Cg-Tg(CAG-DsRed⁺MST)1Nagy/Jas* mice as described in this protocol (Steps 1.1-1.9). In these transgenic mice, the chicken beta actin promoter directs the expression of the red fluorescent protein variant DsRed. MST under the influence of the cytomegalovirus (CMV) immediate early enhancer facilitating the tracking of implants.

To prevent tissue rejection, isogenic individuals with genotype: *C57BL/6-Tg(UBC-GFP)30Scha/J* were selected as hosts. In these transgenic mice, the human ubiquitin C promoter directs the expression of enhanced green fluorescent protein (GFP) in all tissues allowing for a clear differentiation between the recipient and donor tissue. The action of the human ubiquitin C promoter leads to differential GFP expression patterns in certain hematopoietic cell types. In particular, T cells present a 2-fold higher GFP expression levels than CD19⁺B220⁺ B cells or other peripheral blood cells which may facilitate their identification by quantitative sensitive techniques such as flow cytometry, without any needs for additional labeling. Furthermore, fluorescent expression in these mice is observed in adult animals, as well as in all embryonic stages and it is uniform within a cell type lineage granting marker stability.

The pattern for thymus trimming shown in **Figure 1A** follows previous descriptions of functional thymus fragments implanted in the kidney capsula²³. This trimming procedure allows for a reduction of the total volume of tissue to be implanted while preserving the functional histological basic units of the organ (cortex, medulla and cortico-medullary junction (CMJ) blood vessels). The notable difference in the size between newborn thymus (**Figure 1A**, left) and thymus from one-week old mice (**Figure 1A**, right) should be taken into account for trimming, as the space available to fit implants in the anterior chamber of the mouse eye is restricted. The results presented here include the implants obtained from newborns only. **Figure 1B** shows a macroscopic image of a thymic segment implanted in the anterior chamber of a GFP mouse eye, accommodated to preserve animal vision. Histological details of the implant in reference to the cornea (GFP tissue adjacent to RFP implant) can be observed in the 3D reconstruction of confocal images in **Supplementary Video 1**. The engraftment to the iris is shown in **Video 1** and **Figure 2**.

Figure 1C and **1D** respectively illustrate bright field and fluorescence images of the same area which may serve for a double-macroscopic follow up of tissue growth and involution in continuation studies. **Figure 1C** also illustrates the vascularization of the implanted tissue which should uniquely allow for studying thymus physiology dependent on organ blood supply along time.

The vascularization of the inserted thymic fragments occurred within 72h post-implant. The rapid vascularization process is in good agreement with the large amount of blood vessels found in the iris and the richness of the thymus in cytokine production. These characteristics enable fast engraftment of inserted thymi. The vascularization of implants is illustrated in **Video 2**, showing the engraftment of thymic blood vessels with the iris of the host individual (GFP tissue adjacent to RFP implant), therefore, confirming that the model presented here, in fact, allows for noninvasive continuous monitorization of vascularized thymus at the microscopic level. The use of LSM to visualize endogenous thymus or thymus previously implanted in different anatomic locations is restricted by tissue opacity and tissue thickness of over 1 mm. The anterior chamber of the eye is considered as a "window" to the organism facilitating the observation of implanted tissues at the microscopic level.

Here, it is shown that the model developed by our group allows the visualization of transmigration of the cells from the blood torrent (**Video 2**) into the implanted thymus (presumably progenitors); for the tracking of the contacts of GFP progenitor cells incoming into the thymic implant with epithelial and stromal thymic resident cells (RFP-labeled) during differentiation and selection processes (**Videos 3** and **4**) and, also, for the egress of cells (presumably mature T-cells) into blood vessels (**Video 5**).

The approach we describe thus represents the "*proof of principle*", based on recordings taken between 72 h and one month post-implant from six individuals transplanted with the segments from three independent newborn donors, for an animal model that permits *in vivo* real time tracking of thymocyte maturation in a vascularized thymic fragment by noninvasive procedures along time, and as such, the protocol might need further refinements. This model could be further worked out making use of transgenic animals harboring labeled thymocyte molecular markers, for example, as fluorescent fusion products, thus permitting direct visualization of T-cell maturation intermediates. The system may help resolving controversies and respond incognitas linked to thymocyte differentiation and selection events among other.

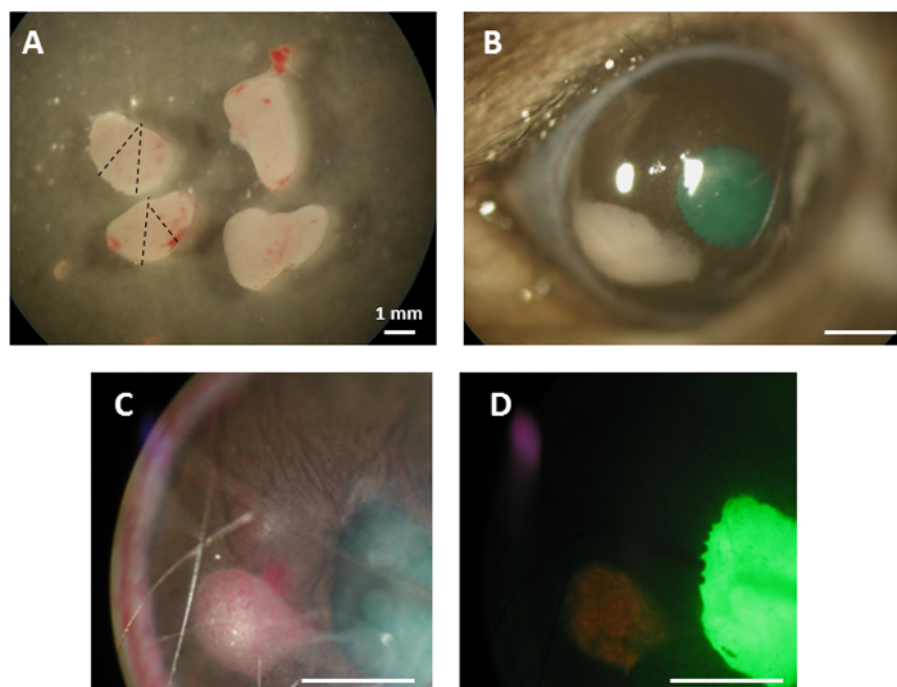


Figure 1. Sectioning of donor newborn thymic lobes and transplant into the anterior chamber of mice eye. (A) Newborn (left) and one week post-natal (right) thymi images. Dash lines on newborn thymus represent guides to prepare sections for transplantation into the anterior eye chamber of mice to estimate the maximum number of transplants from one donor. (B) Representative image showing newly implanted thymus. (C) Representative image of grafted, vascularized thymus 2 weeks post-implant. (D) Fluorescence image of thymus graft shown on (C). Scale bar is shown (1 mm). [Please click here to view a larger version of this figure.](#)

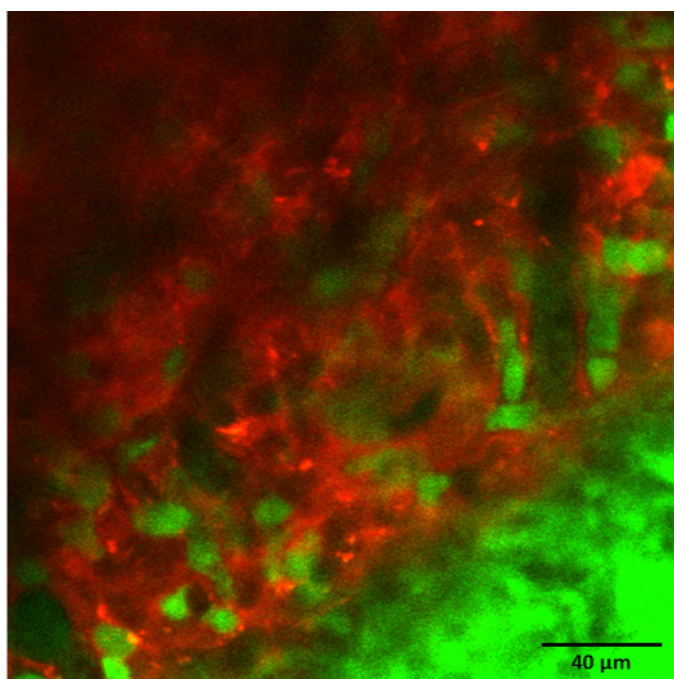
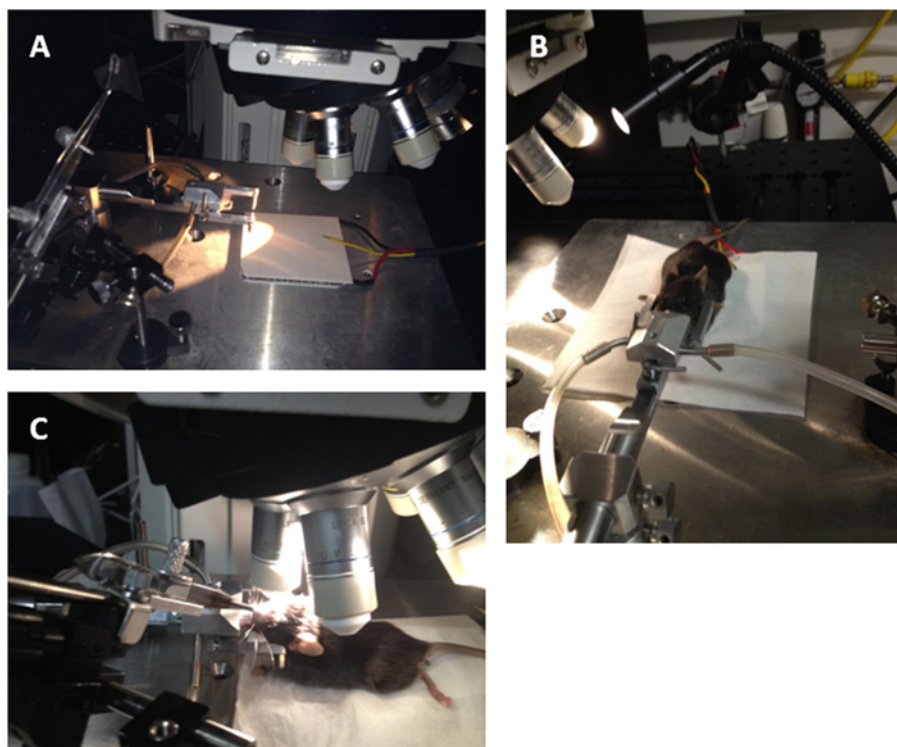
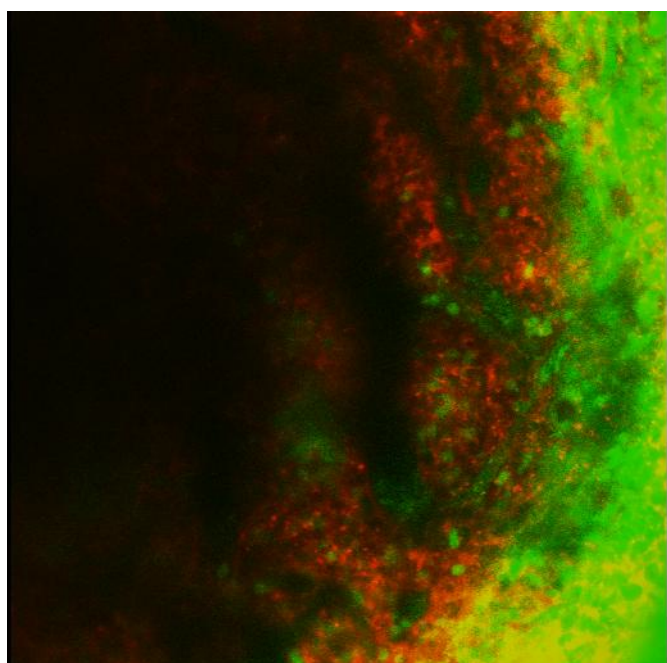


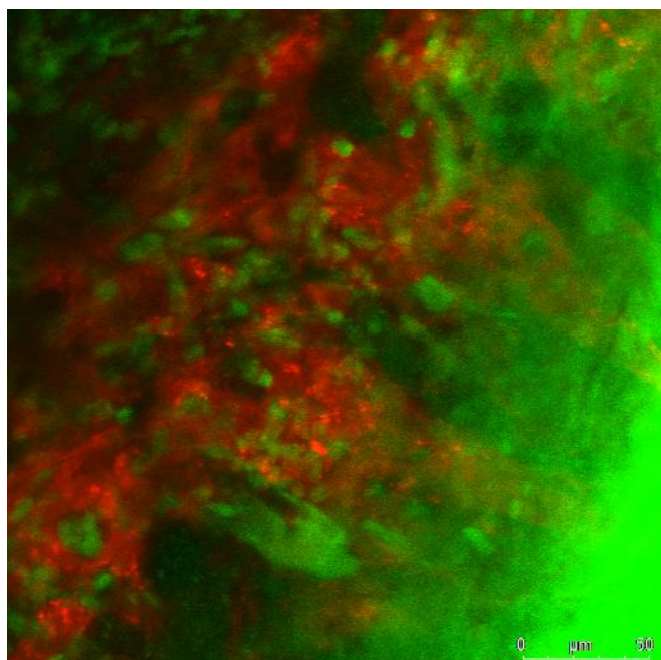
Figure 2. Confocal image showing the detail of thymus segment (red) engraftment in the iris (green). Image obtained with a microscope, using the following settings: 488 and 561 nm excitation/500 and 575 nm emission wavelengths, 512x512 pixel resolution and 40X dipping objective with long working distance. Scale bar is shown (40 μm). [Please click here to view a larger version of this figure.](#)



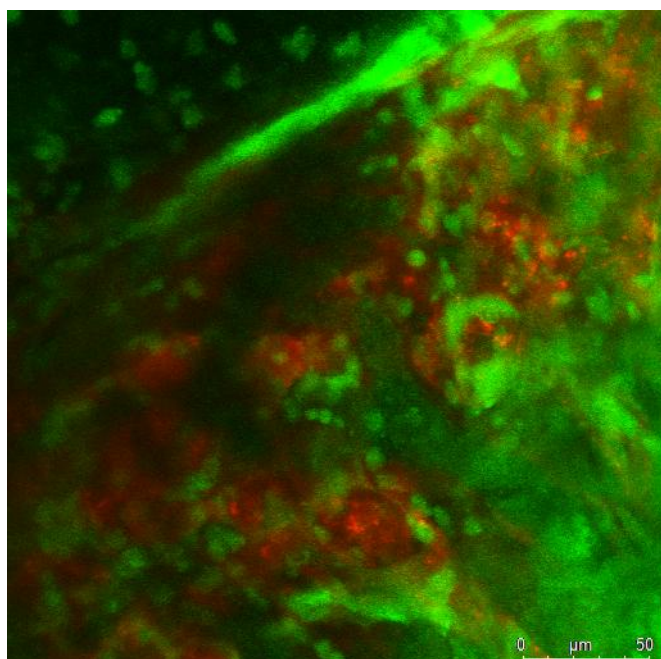
Supplementary Figure 1. Setup for confocal imaging of thymic graft using 3D single photon fluorescence laser confocal microscopy. (A) Heatpad, stereotaxic headholder and gas mask details. (B) Set up of the mouse holding the thymus graft for LSM. [Please click here to view a larger version of this figure.](#)



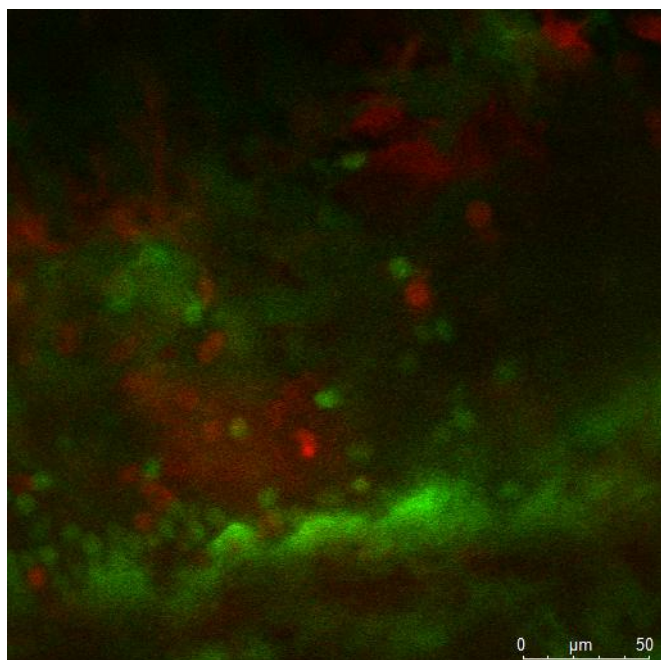
Video 1. *In vivo* imaging of thymus implants in the anterior chamber of the mouse eye. [Please click here to view this video.](#) (Right-click to download.)



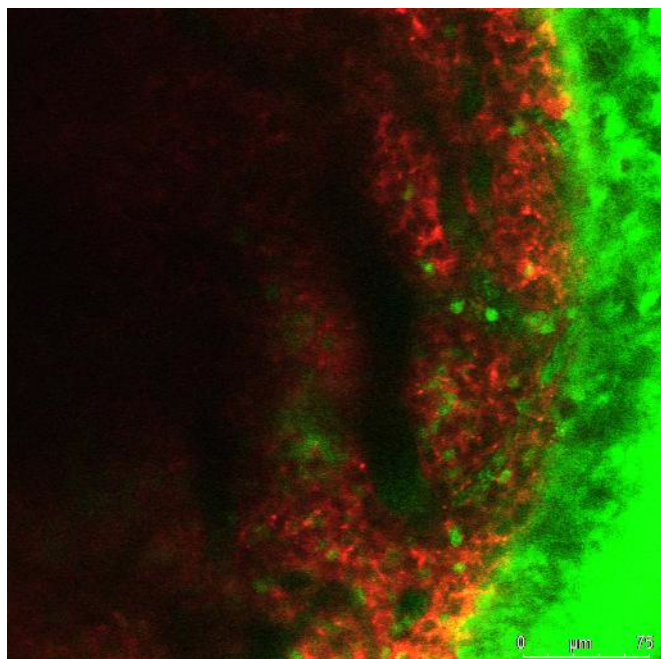
Video 2. Recruitment of presumable thymus settling progenitors (TSP) from the blood stream (GFP) into the thymic parenchyma (RFP). [Please click here to view this video.](#) (Right-click to download.)



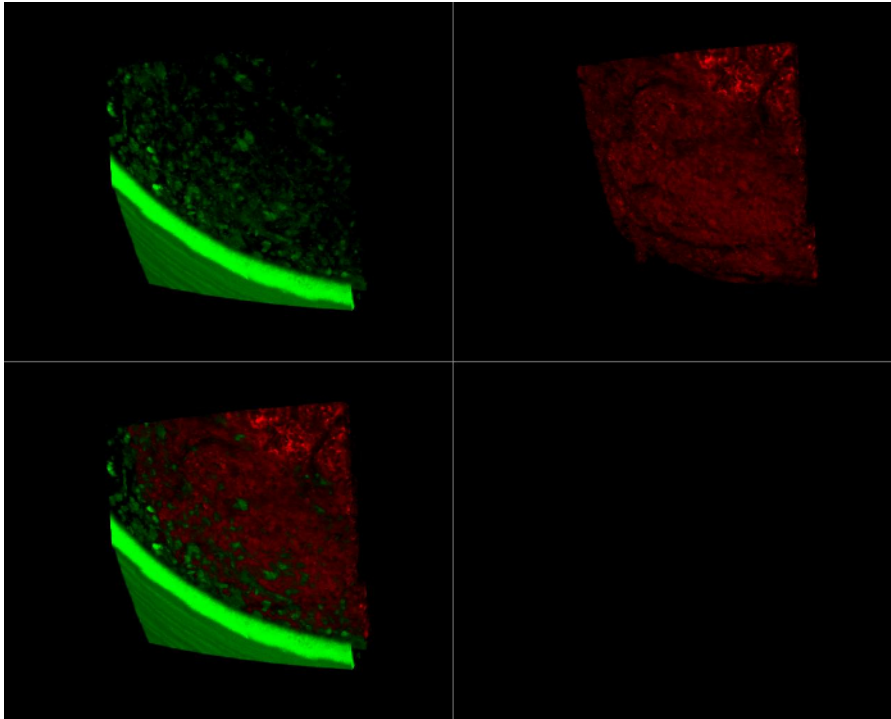
Video 3. Stochastic movement of thymocytes (early thymic progenitors or ETPs) in the cortex. [Please click here to view this video.](#) (Right-click to download.)



Video 4. Interaction of thymocytes (GFP) with thymic stromal cells (RFP) either cTECs (cortical thymic epithelial cells) or mTECs (medullary thymic epithelial cells). [Please click here to view this video.](#) (Right-click to download.)



Video 5. Real time egress imaging of potential mature T-cells. [Please click here to view this video.](#) (Right-click to download.)



Supplementary Video 1. 3D-*in vivo* imaging of thymus implants in the anterior chamber of the mouse eye. Please click here to download this file.

Discussion

Due to the importance of the T-cell maturation process for individual immune competency⁴ and the presumed impact of precursor cell dynamics on mature T-cells produced by the thymus^{2,3}, extensive efforts have been invested to develop alternatives to the classical fixed tissue snapshot approach.

Although tissue slices and other explants are clearly superior in reproducing tissue architecture than monolayers or aggregate thymic organ cultures^{14,15}, the absence of a connection to the vasculature limits their use to study the steps of entry or TSP (translocation of progenitors across blood vessel endothelial cells barrier) and exit (translocation of mature T-cells across blood vessel endothelial cells barrier) from the organ. As it is hypothesized that the dynamics of T-cell contacts with other cells, including thymic stromal cells, and their motility across microenvironments within the cortex and the medulla determine their differentiation status and the process of subpopulation selection (positive and negative)^{2,3,4,5}, and since these processes may depend on TSP and mature T-cell egress events, a model that includes the connection to the individual's blood flow system would be desirable.

Intradermal and kidney capsule implants of the thymus allow for this upgrade^{17,18}. However, they constitute invasive methods with imposed limitations to imaging by TPLSM due to the deep position of the implant within the body or the presence of opaque layers of tissue above the graft. The adaptation of the mouse anterior eye chamber, previously used as a "window"^{21,22} to longitudinally monitor the performance of several tissues, as a site to track T-cells within a vascularized thymus provides several advantages versus other transplantation strategies. On the one side, the surgery is simpler because no sutures are required, leading to faster recovery of host animals. On the other side, the observation of implants does not require an additional surgery as it happens for kidney capsule implants, allowing for repeated recordings from the same animal. In addition, the particular anatomical characteristics of the eye allow to easily modulate regulatory inputs on the implanted tissue not only systemically but also locally. As previously described, with this approach, substances can be applied onto the eye topically or injected into the anterior eye chamber. It also represents an easy option to introduce substances or cells directly into the thymus (*i.e.*, progenitors or maturation intermediates labeled *ex vivo* and adoptively transferred), which has traditionally been a highly complex procedure due to the thymus anatomic location^{24,25}. Furthermore, the perfusion of the anterior chamber permits the exchange of the aqueous humor or loading of the graft by diffusion with fluorescent indicators, as previously shown for other type of implants²⁶.

The approach that more faithfully allows the study of thymus physiology is provided by the intravital imaging of transgenic medaka fish expressing fluorescently labeled lymphocyte markers. The transparency of medaka during development and throughout life in many medaka strains makes this organism advantageous for monitoring cellular dynamics *in vivo*²¹. The low number of motile thymocytes observed in medaka (29%)¹⁸ in reference to mouse's (95%)¹³, in addition to differences in motility rates between these two organisms, however, suggests that the cold blood smallest vertebrate with an adaptive immune system may be limited at revealing particular mechanistic kinetics of higher vertebrate T-cell maturation events.

One limitation of the use of the anterior chamber of the mouse eye as the site for thymus transplantation is space restriction. Long-term studies might be, therefore, compromised; particularly for newborn or embryonic tissues with enlarged growth potential. In this sense, we must state that although newborn implants presented with apparent tissue expansion after the first week post-implant in the mouse eye (data not shown), the enlargement did not seem to put the integrity of the eye at risk up to two months post-implant. Nevertheless, it should be of interest to evaluate

whether the tissue environment of the implant (eye chamber, kidney capsule or dermis) influences implant's growth and/or vascularization before the adequacy of the approach can be established for defined applications.

The large incision required on the cornea to allow the passage of the thymic fragment to the anterior eye chamber leads occasionally to some hazing at this level (data not shown), most likely related to certain degree of fibrosis associated to the healing process. Attention is demanded on Step 2.3.6 of the protocol to ensure that if hazing occurs will minimally interfere with the downstream imaging steps. As the incision is made at a lateral level, neither the implant nor the hazing should significantly affect animals sight performance.

The need for tissue trimming to make the thymus fit into the eye chamber appears to be a disadvantage if compared to other anatomic sites that do not require altering the anatomic integrity of the organ. Nevertheless, the kidney capsule approach has shown that the fragments of the thymus containing, both, cortex and medulla obtained by similar dissection guidelines to those shown in **Figure 1**, become fully functional as they can reconstitute T-cell mediated immunity in immunodeficient thymeless hosts¹⁸. This argues in favor of intact functional traits of this type of thymus segments. Also, the protocol described here stresses that the isolated thymus must be kept in cold (Step 1.9) and only trimmed right before implant (Step 2.3.2) to minimize cold ischemia and tissue exposure towards optimizing tissue engraftment.

An additional potential limitation of the approach resides in the composition differences between the aqueous humor in the anterior chamber of the eye and blood plasma, issue that needs to be kept in mind when interpreting observations made at this anatomic location. Previous studies with other tissues, however, have not reported any obvious effects on normal graft function by the local eye environment²². Although the experience with the model presented here is restricted to a few individuals (N = 6 and N = 3 for recipients & donors, respectively) at present, we did not observe any major alterations associated to implant site in the case of thymic implants in any of the individuals either.

The protocol presented here constitutes a *proof-of-principle* for the study of intravital longitudinal imaging of thymocyte dynamics by implanting thymic tissue into the anterior chamber of the mouse eye. Due to the selection of immunocompetent recipients, the system did not allow questioning the systemic function of the graft. Future studies may build on this approach by choosing different donors and/or recipients and/or by subjecting the hosts to irradiation and bone marrow (BM) transplantation according to particular study designs. For example, the choice of SCID (severe combined immunodeficiency) mice as recipients eliminates the interference of endogenous T-cells, and the choice of transgenic mice donors (thymic implant and/or BM) presenting T-cell markers, DC markers, thymic stromal cell-markers, *etc.*, individually or in combinations should permit direct *in vivo* tracking of cellular subpopulations. Alternatively, subpopulations of cells in the anterior eye chamber of mice could be tracked, as previously described, by *in vivo* fluorescence cytolabeling²⁶.

One major advantage of the model presented here is that it allows for studying progenitor and mature T-cells translocation from and towards blood vessels. Thus, it should be relevant to determine if the rapidly acquired vascularization of the thymus after being implanted in the anterior eye chamber recapitulates the endogenous vasculature. In favor of this possibility, it should be mentioned that the neovascularization in the anterior mouse chamber seems to be dictated by the graft^{18,20} rather than by the recipient and therefore the connections to the iris vasculature should preserve the integrity of endogenous thymus cortico-medullary junction (CMJ) domain.

An additional advantage comes from the fact that the system allows the evaluation of two independent implants (two anterior eye chambers) on identical individual background (a single host). Studies requiring combinations of tissues may also find it advantageous to use this system.

As the thymus plays an important role in autoimmune and immune deficient pathologies, this approach could be used to address a myriad of potential questions. In particular, the system should allow macroscopic in addition to intra-organ imaging for the progression of thymus involution. It may also serve to study transplantation tolerance events and immune dysfunctions related to infections. It is likely that the fine dissection of mechanisms governing thymocyte maturation will provide new clues for the design of additional therapeutic strategies targeting developing T cells.

Transplantation of the thymus to the anterior chamber of the eye takes less than 45 min, including neonatal thymus isolation and trimming for each host individual with implants in either eye. *In vivo* imaging recordings require 1 to 5 h, depending on monitored features.

Disclosures

The authors have nothing to disclose.

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