Published in final edited form as:

Nat Protoc. 2017 August ; 12(8): 1513–1520. doi:10.1038/nprot.2017.045.

Murine chronic lymph node window for longitudinal intravital lymph node imaging

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Abstract

Chronic imaging windows in mice have been developed to allow intravital microscopy of many different organs and have proven to be of paramount importance in advancing our knowledge of normal and disease processes. A model system that allows long-term intravital imaging of lymph nodes would facilitate the study of cell behavior in lymph nodes during the generation of immune responses in a variety of disease settings and during the formation of metastatic lesions in cancer-bearing mice. We describe a chronic lymph node window (CLNW) surgical preparation that allows intravital imaging of the inguinal lymph node in mice. The CLNW is custom-made from titanium and incorporates a standard coverslip. It allows stable longitudinal imaging without the need for serial surgeries while preserving lymph node blood and lymph flow. We also describe how to build and use an imaging stage specifically designed for the CLNW to prevent (large) rotational changes as well as respiratory movement during imaging. The entire procedure takes approximately half an hour per mouse, and subsequently allows for longitudinal intravital imaging of the murine lymph node and surrounding structures for up to 14 d. Small-animal surgery experience is required to successfully carry out the protocol.

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Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

AUTHOR CONTRIBUTIONS E.F.J.M. and H.-S.J. developed the protocol. E.F.J.M. and T.P.P. wrote the manuscript. E.F.J.M. and E.R.P. performed the experiments, including supplementary video acquisition. C.B. and B.J.V. performed intravital imaging and processing of optical coherence tomography. E.F.J.M., E.R.P. and C.B. prepared in-text figures and supplementary videos. T.A.R. designed, developed and prepared the schematics presented in the supplementary figures. T.P.P. supervised all authors who participated in this project. All authors contributed intellectually to, and reviewed, the manuscript.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

INTRODUCTION

Our protocol describes the surgical preparation and implantation of a chronic lymph node window (CLNW) into mice¹, as well as the design of the titanium CLNW frame, processes for using the CLNW with intravital microscopes, including multiphoton microscopy (MPM), and advice for postprocedural follow-up. The subiliac (inguinal) lymph node of the mouse, which is situated in the fold of the flank (regio inguinalis) cranial to the thigh musculature², is revealed by this window preparation.

Ex vivo studies and intravital microscopy of the dynamic microenvironment of the lymph node have expanded our understanding of immune cell interactions^{3–8}. This pioneering microscopy work used acute lymph node preparations that allowed lymph node imaging over several hours. However, to study the development of lymph node metastasis and immune cell trafficking in adaptive immune responses, longitudinal imaging is needed over days and weeks. Previously, an internal lymph node window was created to study the progression of lymphoma⁹. However, this method required successive surgeries to expose the imaging window. Motivated by these seminal methods, we developed the CLNW surgical preparation¹ to facilitate imaging of lymph node anatomy, cancer cell growth and immune cell trafficking over the course of 14 d.

Applications of the method

The CLNW was designed to be used in conjunction with intra-vital microscopy. Intravital microscopy of secondary lymphoid organs has proven valuable in understanding immune cell function and activation^{3,7,8}, as well as malignancy^{1,9}. Other applications may include studying or labeling of blood and lymph to understand fluid and antigen flows in the lymph node^{10,11}, as well as immune and tumor cell trafficking. To achieve the latter application, murine tumor cell lines can be administered to the mice before imaging to generate spontaneous inguinal lymph node metastasis. MPM has proven useful because of its superior tissue penetration *in vivo*^{12,13} as compared with other fluorescence-based techniques of similar spatial resolution. Images can be created in the CLNW from depths of several hundred microns using MPM *in vivo*¹. Optical coherence tomography (OCT)^{14–16} can be used for deeper imaging of the lymph node in the CLNW, although this technique currently has a lower spatial resolution.

Comparison with other models that use chronic windows

There are multiple chronic windows available for intravital microscopy, and each is optimized for its targeted organ. The mammary fat pad window¹⁷ has been developed to study healthy and tumorigenic mammary tissue in mice¹⁸. The dorsal skin-fold chamber is used to image the subcutaneous tissue, as well as tumor growth in the skin^{19,20}. The cranial window, created by replacing a portion of the mouse skull with a glass coverslip that is permanently secured to the skull with glue, is used to image the brain²¹. Other chronic windows developed for imaging the nervous system include a mouse spinal cord window²² and a rat peripheral nerve window²³, which transposes the sciatic nerve to surrounding muscle tissue, providing stable access to this nerve. Last, an abdominal imaging window has been developed to chronically image abdominal organs²⁴. Our CLNW is comparable to

these windows in concept. To image the inguinal lymph node using intravital microscopy as previously described in Jeong *et al.*¹, we modified the chronic mammary fat pad window model. Similar to the mammary and abdominal imaging windows, the CLNW allows for coverslip replacement, giving access to the tissue (e.g., for cleaning or experimental manipulation) without having to adjust the surgical preparation. This capability allows better image acquisition over time. Similar to other window chambers, the CLNW uses titanium for its known biocompatibility and favorable strength-to-weight properties. The CLNW and its schematic design are shown in Figure 1 and Supplementary Figures 1–3, respectively.

Limitations

The CLNW implantation will impair leg movement in mice weighing less than ~25 grams (age 6–8 weeks). The procedure is considered major surgery, and therefore animals need time to recover before additional anesthesia is administered for imaging. We allow the animal to recover for 48 h after surgery before imaging begins, during which time we administer acetaminophen chronically in accordance with recommendations from the Massachusetts General Hospital (MGH) Institutional Animal Care and Use Committee (IACUC).

The inguinal lymph node undergoes minimal morphological, cellular and biochemical changes during the first 14 days after CLNW implantation, as described by Jeong *et al.*¹. Briefly, local inflammation and vascular remodeling is microscopically absent in the physiologic lymph node post CLNW implantation, as shown by imaging and immunofluorescence staining. In addition, lymphotoxin- β receptor (LTbR) and tumor necrosis factor (TNF) expression remains low for 2 weeks postoperatively (see Supplementary Fig. 1d in Jeong *et al.*¹). A transient increase in interferon gamma (IFN γ) was noted 2 d postoperatively, but IFN γ levels returned to baseline shortly thereafter. Together, these data indicate that some transient regional inflammation due to the surgery is to be expected at day 2 postoperatively and is resolved by day 4. Thereafter, stable levels of inflammatory cytokines and little to no changes in lymph node architecture are to be expected. On the basis of longitudinal second-harmonic generation (SHG) imaging of collagen in the CLNW, fibrillar collagen content or organization does not change because of the presence of the CLNW. Further, we characterized the immune cell populations by flow cytometry data in Balb/c mice on days 2, 7 and 14 after CLNW implantation in comparison with mice without CLNW implantation (Supplementary Fig. 4). These data show no significant changes in the total CD45⁺ cell populations through day 7 ($P > 0.05$), although by day 14 there are differences in the number of CD45⁺ cells, as well as in many CD45⁺ subpopulations (Supplementary Fig. 4c). After 14 d, in animals in which all subcutaneous fat covering the lymph node was removed, mild inflammation and gradual loss of perfusion in cortical blood vessels became apparent, and therefore it is not recommended to continue to image in these animals.

This preparation provides visual access predominantly to the lymph node cortical region. With sufficient practice, a surgical success rate of 100% is achievable and substantial movement of the lymph node over days can be prevented, although slight rotational changes cannot always be avoided. Therefore, we suggest using an imaging method that can acquire

images in both the horizontal and vertical axes to ensure you will always be able to identify the same imaging region.

Experimental design

Any mice aged >8 weeks can be used. Murine tumor cell lines can be administered before imaging (Step 1) to generate spontaneous inguinal lymph node metastasis in various immune-competent mouse strains, if desired.

For optimal microscopy, the optical clarity of the CLNW is critical. The coverslip can be replaced when tissue exudate impairs image quality without altering the surgical preparation. This is accomplished based on the design of the titanium frame, in which the coverslip is secured through the use of a tension C-ring. When employing imaging modalities using water-immersion objectives, the coverslip should be sized using precise dimensions to prevent leakage of water. When mounted to an imaging stage, the titanium frame prevents respiratory movement during imaging, permitting high-resolution imaging *in vivo*.

The titanium frame can cause skin damage on the ventral side; therefore, a layer of surgical tape wrapped around the side of the ventral titanium frame will prevent unnecessary harm to the animal and improve the experimental conditions (Fig. 2). For longer-term experiments, the coverslip may cause superficial damage to the lymph node if the subcutaneous fat overlying the lymph node is removed; this will become apparent by the gradual loss of perfusion in cortical blood vessels starting after 14 d. Depending on the question to be answered, and keeping in mind the limitations of the imaging modality used, the direct exposure of the lymph node to the coverslip can be adjusted by altering the timing of when the subcutaneous fat is removed and the amount of fat removed. Complete removal of subcutaneous fat covering the lymph node will allow for at least 14 d of imaging, and all experiments described in this protocol were performed with complete removal of the subcutaneous fat covering the lymph node.

We have developed an imaging stage specifically designed for the CLNW to prevent (large) rotational changes, as well as respiratory movement (Supplementary Figs. 5–10). Sequential imaging of the same location over multiple days is achieved at the macro scale by returning to the same position within the CLNW, as the lymph node will remain in place if the CLNW is implanted correctly. If needed, a small ink mark on the cover glass can be made to circumscribe the previous imaging area. At the micro scale, the same location can be found by tracking anatomical landmarks such as blood vessels and collagen structure (Fig. 3). This works well for frequent imaging sessions in tissue not undergoing large-scale remodeling.

In lymph nodes with extensive vascular remodeling, identifying collagen structure by SHG imaging or by the locations of larger unchanged blood vessels can help in imaging identical regions over time (Fig. 3). In lymph nodes with extensive expansion (e.g., caused by experimental inflammation models or metastasis), the coverslip will keep the lymph node in its same general orientation with respect to the imaging window. This is important for lymph nodes undergoing large changes owing to inflammation, adaptive immune response or growth of metastatic cancer. With these scenarios, the ability to measure micro-level changes is less meaningful as compared with the ability to measure massive changes in cellular

content and general architecture of the node. The CLNW allows for the kinetics of these processes to be monitored in a more efficient and controlled manner than could be achieved by looking collectively at cohorts of similarly treated animals at staggered time points.

Animals are not expected to have reduced vital signs or body weight. The researcher should be experienced with the surgery to obtain reproducible results. For experiments in which pharmacological treatments are included, animals with and without CLNW should be compared to examine possible overall health effects of the CLNW and analgesics. When examining effects using *in vivo* imaging, treated CLNW-implanted animals should be compared with nontreated CLNW-implanted animals.

MATERIALS

REAGENTS

▲ **CRITICAL** All reagents and equipment can be substituted with appropriate alternatives from other manufacturers.

- Mice: preferably aged 8–10 weeks or older and weighing at least 25 g. Strain and sex are dependent on the experiment. We have successfully used C3H mice, BALB/c mice, C57BL/6 mice, nude mice and mice from a mixed background. All mice were bred and kept in our animal facility ! **CAUTION** All animal studies must be reviewed and approved by the relevant local IACUC and must conform to all relevant ethics regulations. We obtained institutional approval for this procedure from the MGH IACUC.
- (Optional) cell lines: 4T1 mammary carcinoma cells (ATCC, cat. no. CRL-2539), B16-F10 melanoma cells (ATCC, cat. no. CRL-6475) and SCCVII squamous cell carcinoma cells (originally established in the Edwin L. Steele Laboratories, MGH). Cell lines can be stored in liquid nitrogen, aliquotted in cryogenic storage vials ! **CAUTION** The cell lines used in your research should be regularly checked to ensure that they are authentic and that they are not infected with mycoplasma.
- DMEM (Thermo Fisher Scientific, cat. no. 11965118). This compound can be stored at 2–8 °C for 12 months from the date of manufacture, if protected from light
- FBS (Thermo Fisher Scientific, cat. no. 10437036). This compound can be stored at –5 to –20 °C until the date of expiration
- Buprenorphine hydrochloride (McKesson, cat. no. 1492602). This compound can be stored at room temperature (20 °C) until the date of expiration ! **CAUTION** This substance may cause prolonged respiratory depression. Wear protective clothing to avoid contact or inhalation. Buprenorphine is a controlled substance and should be handled accordingly.
- Acetaminophen (McKesson, cat. no. 633792). This compound can be stored at room temperature until the date of expiration. ! **CAUTION** Although correct dosing should be safe, overdosing can result in substantial morbidity and

mortality. To prevent overdosing, apply only to drinking water at 1 mg/ml. Do not inject or gavage.

- Ketamine (McKesson, cat. no. 409205310). This compound can be stored at room temperature until the date of expiration ! **CAUTION** Ketamine may cause prolonged respiratory depression. Ketamine is a controlled substance and should be handled accordingly. Inject it i.p. or s.c.
- Xylazine (Webster, cat. no. 200204.00). This compound can be stored at room temperature until the date of expiration ! **CAUTION** Xylazine may cause prolonged respiratory depression. Xylazine is a controlled substance and should be handled accordingly. Inject it i.p. or s.c.
- Ophthalmic ointment (Patterson, cat. no. 07-8882572). This compound can be stored at room temperature until the date of expiration
- Hair removal cream (CVS Pharmacy, cat. no. 339826). This compound can be stored at room temperature until the date of expiration
- Glue (Krazy Glue, Staples, cat. no. 654643)
- Tetramethylrhodamine-dextran (Thermo Fisher Scientific, cat. no. D7139). For long-term storage, create an aqueous 1% (wt/vol) solution in sterile phosphate-buffered saline, divide into 1-ml aliquots and freeze them at -20°C . This compound can be stored -20°C until the date of expiration ! **CAUTION** Avoid repeated freezing and thawing. This compound can be stored at $2-6^{\circ}\text{C}$ for several weeks, if protected from light.

EQUIPMENT

- Gauze sponge (Owens & Minor, cat. no. 4352NON25334)
- Scalpel, size >10 (Fisher Scientific, cat. no. 08-916-5A)
- Custom-made titanium frame (CNLW frame; Fig. 1; Supplementary Fig. 1)
- Custom-made titanium ring (Fig. 1; Supplementary Fig. 2)
- Tension C-ring insert (McMaster, cat. no. 91580A146)
- M2x6 socket cap screws (McMaster, cat. no. 91290A013)
- M2 nuts (McMaster, cat. no. 94150A305)
- 5-0 polypropylene sutures (Owens & Minor, cat. no. X698G)
- Sterile cotton swabs (Owens & Minor, cat. no. 1314WOD1002)
- Coverslips, 11.7 mm (Fisher Scientific, custom-made)
- Insulin syringe, 1 ml (BD Micro-Fine, cat. no. 329412)
- Microdissection scissors, 3.5-inch (Roboz Surgical, cat. no. RS-5880)
- Scissors (Roboz Surgical, cat. no. RS-5960)
- 2 Dumont forceps, no. 5 (WPI, cat. no. 500233)

- Heating pad (Shor-Line, cat. no. 912.1000.05)
- Needle holder (Symmetry Surgical, cat. no. 36-1006)
- Surgical tray (WPI, cat. no. 500254)
- Nitrile gloves (Fisher Scientific, cat. no 19-166-107)
- Cautery (Cardinal Health, cat. no. 65410-185)
- Surgical bright-field microscope (Olympus America, cat. no. BIO-16700834)
- Preferred imaging system. For multiphoton imaging, there are many commercially available upright microscopes. We use a custom-built multiphoton laser-scanning microscope (adapted from the Olympus 300; Optical Analysis Corp.) and a broadband femtosecond laser source (High Performance Mai Tai, Spectra-Physics)
- Shaving device (Patterson, cat. no. 07-858-3431)
- Laminar flow hood (DEL Lab Products, cat. no. 30909)
- Surgical tape (Owens & Minor, cat. no. 4509015270)
- Sterile saline for injection (McKesson Medical-Surgical, cat. no. 239930)
- Sterile water (Owens & Minor, cat. no. 0706-2F7113)
- Scale, capable of measuring mouse weight (Fisher Scientific, cat. no. 01-919-33)
- Nut driver, M3 size (McMaster, cat. no. 52965A23)
- Ring pliers (Lawson, cat. no. 95436)
- LabVIEW (National Instruments, <http://www.ni.com/download-labview/>)

REAGENT SETUP

Anesthesia—Mix ketamine and xylazine with sterile saline to obtain a concentration of 100 mg/ml for ketamine and 10 mg/ml for xylazine in a single dilution. The ketamine–xylazine mixture can be stored for up to 3 months at room temperature.

EQUIPMENT SETUP

Surgical station—The surgery must be performed in an aseptic working environment. Therefore, we recommend performing surgery in a laminar flow hood with sterile equipment inside while practicing sterile surgical technique.

Surgical kit preparation—All items must be sterilized before surgery. The surgical tray, microdissection scissors, scissors, two Dumont no. 5 forceps, the needle holder and all CLNW components, including coverslips, should be sterilized.

Animal housing—All animals should be caged separately postoperatively. Food should be placed directly on the bedding and a cage discouraging climbing is preferred to prevent the

animal getting stuck owing to the titanium chamber. Mouse well-being should be monitored per the relevant guidelines and regulations in place.

PROCEDURE

Preparation before the day of surgery ● TIMING 5–60 min

- 1| Murine tumor cell lines can be used to generate spontaneous inguinal lymph node metastasis in various immune-competent mouse strains. To set up this model, implant 4T1 mammary carcinoma cells in the fourth mammary fat pad of BALB/c mice or implant B16-F10 melanoma cells subcutaneously in the flank (caudal of the inguinal lymph node) of C57BL/6 mice. Alternatively, SCCVII squamous cell carcinoma cell from the buccal mucosa can be grown subcutaneously in the flank of C3H/HeJ mice. Implant each cell line at a concentration of 0.1×10^6 cells/animal in 30 μ l of DMEM with 10% FBS. After primary tumors metastasize to lymph nodes (~7–10 d), the CLNW can be implanted. ! **CAUTION** Cell line implantation and subsequent CLNW implantation must be reviewed and approved by the relevant authorities. Our procedure was reviewed and approved by the IACUC at MGH.

▲ **CRITICAL STEP** This step is optional, depending on whether spontaneous metastatic lymph nodes are to be studied.
- 2| Add 1 mg/ml acetaminophen to drinking water 2 d preoperatively. Sterilize all necessary equipment.

Preoperative preparations ● TIMING 10 min

- 3| Place the mouse on the scale to weigh it. Anesthetize the mouse using the ketamine–xylazine mixture (100 mg/10 mg per kg body weight). Maintain the body temperature at 37 °C throughout the procedure using a heating pad at 39 °C. Apply ophthalmic ointment to both eyes.
- 4| Remove hair by shaving the area of the mouse bounded by the femoral region and the hypochondriac region, and the ventral and dorsal midlines (Fig. 2a). Subsequently, apply hair removal cream. Remove the cream after 30–60 s with sterile water and cotton gauze to prevent chemical skin burn.

Surgery ● TIMING 15 min

- 5| Pull a suture through the most posterior of the smaller holes of the CLNW frame. Place the suture above the subiliac lymph node 2 mm from the edge of the regio plicae genus, with the mouse in the supine position, and secure the CLNW in this position with a surgical knot (Fig. 2b,c, white arrow). The lymph node location (Fig. 2b–d, yellow asterisk) can be noninvasively determined by highlighting the lymph node through the skin with bright light from the dorsal side while looking at the ventral side. If the lymph node is not visible, use the nipple of the fourth mammary fat pad to approximate its position. Optimal

positioning of this suture is important for centering the lymph node in the imaging window. The surgical preparation is depicted in Figure 2.

- 6| Place two screws through the most lateral holes in the CLNW and secure them by tightening one nut each to the frame. Starting with the screw closest to the leg, pierce the skin directly above the screw using a scalpel or scissors and place the screw through the hole. Repeat for the second screw (Fig. 2c).

? TROUBLESHOOTING

- 7| Wrap a layer of surgical tape around the side of the second CLNW frame to prevent unnecessary harm to the animal. Place the second CLNW frame on top of the other (Fig. 4), with the skin in between. Cut away tape obstructing the titanium ring opening. Secure this CLNW with two additional nuts, this time only loosely tightening them (Fig. 2d).

! CAUTION Do not overtighten these nuts, as this will impede blood flow to the inguinal region and subiliac lymph node. Check the range of motion of the limb to ensure that the animal can move its limb. The positions of these nuts will be secured in Step 10.

- 8| Remove the suture placed in the dorsal CLNW in Step 5 (Fig. 2b, white arrowhead). Place two or three sutures through each of the holes on the ventral CLNW. Together with the CLNW, these sutures will keep the tissue in place for 14 d. Placing the sutures through one but not both CLNW frames makes it harder for the animal to remove the sutures. Placing these sutures through only the ventral CLNW frame specifically allows for microscopy using water-immersion objectives so that the water will remain in place.
- 9| Place the mouse under the surgical bright-field microscope. Use microdissection scissors to open one layer of ventral skin ~6 mm in diameter (Fig. 2e). If substantial bleeding occurs from the cutaneous blood vessels, use a cautery to stop the bleeding. Usually, the lymph node will be visible after opening the dermis. Any overlying subcutaneous fat can be removed using curved blunt 5.0 forceps.

▲ CRITICAL STEP If the lymph node is not directly visible, carefully explore the mammary fat pad using blunt dissection. Do not use a cautery in the vicinity of the lymph node, nor use forceps directly on the lymph node, as this will inflict irreversible damage.

? TROUBLESHOOTING

- 10| Apply sterile saline to the wound area to prevent tissue dehydration. Place a glass coverslip into the CLNW groove, covering and protecting the lymph node, and secure the coverslip using a tension C-ring (Fig. 2f). Use glue to secure the nuts applied loosely to the screws in Step 7 to prevent CLNW detachment.

Postoperative care ● TIMING 5 min

- 11| Inject 10 ml/kg body weight buprenorphine hydrochloride s.c. once postoperatively before the animal wakes up. Repeat, if necessary, at any point during daily postoperative follow-up if the animal is showing signs of pain or distress, up to twice daily, in line with all relevant guidelines and regulations of the governing IACUC. Monitor daily to make sure that the animal is fully ambulatory, with the CLNW in place, and that it shows no signs of edema caused by the CLNW. Wait 2 d before administering anesthesia for imaging or other experimental procedures.

? TROUBLESHOOTING

Imaging TIMING 15–90 min, beginning 48 h or more after CLNW implantation

- 12| For all imaging procedures, check whether the imaging setup you are using is working correctly, as appropriate for the system being used and according to the manufacturer's guidelines. Then, anesthetize the mouse using a 100/10 mg ketamine–xylazine mixture per kg body weight or by using isoflurane.
- 13| Position the mouse on an imaging stage (Figs. 5 and 6). For fluorescence-based angiography, inject 2,000,000 MW tetramethylrhodamine–dextran (or similar) intravenously.
- 14| If using multiphoton imaging with a broadband femtosecond laser source (used as we described previously^{13,25}), set the laser to ~60 mW on sample laser power at an 820-nm wavelength for tetramethylrhodamine detection before imaging.
- 15| Use the slowest acquisition time to get the best-quality images possible and do not digitally alter gain or offset. Perform *xyz* or *xyzt* (for video) imaging, with *z* (depth) ~20–100 μm . Images from Figure 3 were obtained using Olympus FluoView software with a 25 $\times$ 1.05 numerical aperture (NA) water-immersion objective at 1 $\times$ zoom, stack *z* ~50 μm , 512 $\times$ 512 pixels, and 16-bit. For signal detection, use a 610DF75 emission filter for tetramethylrhodamine (blood vessels), a 535DF43 filter for green (GFP cancer cells) and a 450DF100 filter for SHG imaging in front of the photomultiplier tubes. We use a motorized stage (Prior Scientific, model no. H101) combined with customized automation software (LabVIEW) to obtain mosaic images. Settings were similar for the supplementary videos (Supplementary Videos 1 and 2); small differences are listed in their respective legends. ImageJ (National Institutes of Health (NIH)), or similar image analysis software, can be used to combine data acquired from different photomultiplier tubes. Images with data combined from different color channels can help co-localize different features in your experimental system.

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

● TIMING

Steps 1 and 2, preparation before the day of surgery: 5–60 min

Steps 3 and 4, preoperative preparations: 10 min

Steps 5–10, surgery: 15 min

Step 11, postoperative care: 5 min

Steps 12–15, imaging: 15–90 min

ANTICIPATED RESULTS

For the first 14 d after surgery, clear, high-quality images are expected using the CLNW. The CLNW will also prevent movement artifacts during imaging and will not impede blood or lymph flow if implanted properly. For this purpose, the surface of the glass coverslip can be easily cleaned with ethanol before imaging, or the coverslip can be removed and replaced. As noted earlier, minimal morphological, cellular and biochemical changes are expected (see Supplementary Figs. 1d and 4 in Jeong *et al.*¹) and successive surgeries are not required for exposing the imaging window in the CLNW.

Examples of imaging results after CLNW implantation are shown in Figure 3. The lymph node and surrounding vasculature can be imaged by optical coherence tomography (Fig. 3a). Metastatic Dendra2-expressing 4T1 breast cancer (green) in the inguinal lymph node (Fig. 3b,c) can be monitored longitudinally. Blood vessels were used to repeatedly find the region of interest in the lymph node, allowing the imaging to be centered on the growing metastatic lesion. Blood vessels can be identified by intravenous injection of fluorescently labeled dextran.

To generate metastatic lymph nodes, squamous cell carcinomas or melanomas can be injected into the thigh subcutis or breast tumor cells can be injected into the fourth mammary fat pad of the mouse; these will subsequently metastasize to the inguinal lymph node. Lymphangiography can be used to confirm whether tumors drain to the inguinal lymph node^{1,26}. At the early stages of lymph node metastasis—~7 days post tumor implantation of the 4T1 breast cancer cell line—tumor cells can be visualized in the subcapsular sinus of the lymph node. At later time points, these cells invade the parenchyma, moving toward blood vessels¹. The timing of CLNW implantation can be tailored to the research question and the metastatic behavior of the tumor cell line. Tumor chunk implantation of the primary tumor can be considered to avoid direct spread of tumor cells into the inguinal lymph node from the initial injection. To study immune cell behavior in metastatic lymph nodes, fluorescently labeled immune cells can be adoptively transferred to a tumor-bearing mouse and subsequently imaged in the tumor-draining lymph node using the CLNW.

In addition to monitoring the progression of tumor growth in the lymph node over days, it is possible to perform short-term time-lapse intravital imaging using the CLNW to analyze tumor cell interaction with immune cells and other lymph node stromal cells, as has also been accomplished using prior models^{3,7,8}. Supplementary Video 1 shows a maximum-

intensity project of a 3D lymph node tissue acquired by time-lapse multiphoton microscopy in LysM-GFP transgenic mice. Lymph node neutrophil and monocyte movement (green) in blood vessels (red) and lymph node tissue can be observed. In Supplementary Video 2, time-lapse microscopy was performed at a higher resolution for a high-endothelial vessel in the lymph node that shows GFP-positive cells flowing, rolling and extravasating.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the NIH under award nos. R00CA137167 (T.P.P.), DP2OD008780 (T.P.P.), R01CA163528 (B.J.V.) and R01HL128168 (T.P.P.). Research reported in this publication was supported in part by the Center for Biomedical OCT Research and Translation through grant no. P41EB015903 (B.J.V.), awarded by the National Institute of Biomedical Imaging and Bioengineering of the NIH. National Cancer Institute Federal Share of Proton Income grant CA059267 (T.P.P., B.J.V.) and the Massachusetts General Hospital Executive Committee on Research ISF (T.P.P.) also supported this work. We thank J. Kahn, E. Beech and C. Cui for outstanding technical support.

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Figure 1. CLNW parts and assembly. **(a)** Titanium frame. **(b)** Titanium ring designed to fit perfectly into the center of the titanium frame. **(c)** The complete CLNW, including the titanium frame, ring and the tension C-ring insert. CAD schematics for each individual part are available in Supplementary Figures 1–3.

Figure 2.

Surgical procedure. **(a)** Shave the area of the mouse bounded by the femoral region and hypochondriac region, and the ventral and dorsal midlines (Step 4). **(b)** Pull a suture (white arrow) through the most posterior of the smaller holes of the CLNW and place the suture above the subiliac lymph node (asterisk) (Step 5). **(c)** Pierce the skin directly above the screws using a scalpel or scissors, and place the screws through the holes (Step 6). **(d)** Place the second CLNW on top of the other and secure with two nuts (Step 7). **(e)** Remove the suture placed previously and place sutures through the holes on the ventral CLNW (Step 8), and then open the skin. **(f)** Expose the lymph node using blunt dissection of perinodal fat (Step 9), position the glass coverslip and secure with the tension C-ring. Last, use a small drop of glue to secure each nut (Step 10). The yellow asterisk in **b–d** shows the position of the lymph node. These experiments were approved by the IACUC at MGH.

Figure 3.

Lymph node imaging using a CLNW. **(a)** Optical coherence tomography image of entire lymph node and surrounding vasculature 2 d post CLNW implantation. Gradient from yellow (superficial) to red (deep) indicates the blood vessel depth; lymphatic vessels are shown in blue. **(b,c)** Lymph node MPM images acquired 7 **(b)** and 12 d **(c)** post CLNW implantation, monitoring the growth of cancer cells in a tumor-draining lymph node. Arrowheads indicate identical blood vessel locations, whereas the tumor area remains centered in the imaging region of interest. The blood is labeled with tetramethylrhodamine-dextran (red). The 4T1 tumor cells express Dendra2 (green) and collagen (blue) and are visualized by SHG. **(d–g)** Lymph node harboring metastasized 4T1 tumor cells. At day 4 after tumor cell injection into the mammary fat pad, the CLNW was implanted. SHG imaging of the changes in fibrillary collagen in a single location over time at days 6 **(d)**, 8 **(e)**, 10 **(f)** and 13 **(g)** by MPM is shown. Scale bars, 500 μm **(a)**; 50 μm **(b–g)**. Images obtained with a 25× 1.05 NA water-immersion objective at 1× zoom, 512 × 512 pixels, 16-bit. These experiments were approved by the IACUC at MGH.

Figure 4. Combining of the CLNW frames. Representation of how two CLNW frames connect. The ventral CLNW frame has both a tension C-ring and a coverslip inserted.

Figure 5.

Assembled CLNW imaging stage. The CLNW imaging stage consists of an acrylic base, with springs made from spring steel, which can be adjusted. The CLNW screws are positioned in the predrilled holes in the imaging plate, preventing movement during imaging. The CLNW is gently secured with two spring clamps. Supplementary Figures 5–10 show the schematics for the separate parts.

Figure 6. Imaging setup. For demonstration purposes, the objective is slightly elevated and water was not added between the coverslip and the objective. The tape wrapped around the side of the ventral titanium window and the heating pad below the mouse were removed so that we could clearly show how the CLNW is mounted. This experiment was approved by the IACUC at MGH.

TABLE 1

Troubleshooting table.

Step	Problem	Possible reason	Solution
6	Skin around the screw is too loose, causing the CLNW to change location over time	Hole in the skin is too large	Try using a scalpel instead of scissors to make the hole. Make an incision smaller than the screw width. Gently stretch the skin over the screw to get a perfect fit
9	Surgical damage to the lymph node	Trauma caused by surgical tools	We suggest starting blunt dissection of subcutaneous fat at a side of the lymph node where there is no major blood vessel visible to prevent bleeding. Gently pull this fat aside to find the correct tissue layer. Then, gently pull away the fat covering the lymph node. To prevent pinching and damaging the lymph node, do not place your Dumont forceps directly on top of the lymph node. This will allow for safer removal of the subcutaneous fat located on the top (ventral side) of the lymph node
11	Lymph node position changes over time	Sutures are lost	Animals should be monitored daily postoperatively, and if any sutures are lost the sutures should be replaced to keep the lymph node in place
15	z drift during imaging when using a water-immersion objective	Water evaporated from between the coverslip and the objective	Water can be carefully added during z-stack acquisition if z drift is bothersome. Alternatively, acquire a larger z stack to later adjust for the drift using image analysis software, or change the imaging modality to one that is not dependent on water-immersion objectives