

Submission ID #: 71140

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Project Page Link: <https://review.jove.com/account/file-uploader?src=21697748>

Title: Integrated Protocol for Assessing Meningeal Lymphatics: Cisterna Magna Injection, Lymph Node Imaging, Meningeal Dissection, and Whole-Mount Staining

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Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **YES**

If **Yes**, can you record movies/images using your own microscope camera?

NO

If your protocol involves microscopy but you are not able to record movies/images with your microscope camera, JoVE will need to use our scope kit.

If your microscope does not have a camera port, the scope kit will be attached to one of the eyepieces and **you will have to perform the procedure using one eye.**

JSZ6 Stereo Microscope, Nanjing Jiangnan Novel Optics Co.

If a dissection or stereo microscope is required for your protocol, please list all shots from the script that will be visualized using the microscope (shots are indicated with the 3-digit numbers, like 2.1.1, 2.1.2, etc.).

Scope Shots:

2.10.1

2.10.2

2.10.3

2.10.4

2.11.1

2.12.2

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **YES**

3. Filming location: Will the filming need to take place in multiple locations? **NO**

4. Testimonials (optional): Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **NO**

Current Protocol Length

Number of Steps: 33

Number of Shots: 54

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Baokai Zhang:** Our research focuses on mouse meningeal lymphatic vessel function and morphology assessment methods.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research contexts or systems in which this protocol can be applied.~~

- 1.2. **Baokai Zhang:** This protocol supports neurological disease models, drug delivery, molecular correlation, and translational studies.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Baokai Zhang:** Using this approach researchers can investigate tracer drainage function and dorsal MLVs network morphology in mice.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Baokai Zhang:** The biggest challenge is performing accurate intracisternal injections and careful meningeal dissection while preserving vessel integrity.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.5. **Baokai Zhang:** Following this procedure, researchers can perform RNA and protein extraction, intracranial drug delivery, or analyses in disease models.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee (IACUC) at Shanghai University

Protocol

Videographer: Please always record an audio slate while filming each shot mentioned in the shotlist.

NOTE: SCREEN timestamps for protocol were added at the postshoot stage. Please contact the postshoot note integrator (Sri Lakshmi Priya) for queries regarding lab media.

2. Intra-cisterna Magna Injection and Live Imaging

Demonstrator: Baokai Zhang

- 2.1. To begin, gather the surgical instruments and required buffers [1]. Use blunt forceps to break off the 30 gauge needle and retain the tip [2].
 - 2.1.1. WIDE: Talent walking into the room with a tray of surgical instruments and buffers. *Videographer: Please obtain CU shot as well for this shot.*
 - 2.1.2. Talent using blunt forceps to break off the 30 g needle then holding the detached tip piece.
- 2.2. Using a 30 centimeter length of polyethylene 10 or PE 10 (*P-E-ten*) tube, connect the broken 30 gauge needle to the microinjection needle containing a 30 gauge needle [1].
 - 2.2.1. Close-up of the tubing being connected between the broken needle tip and the microinjection needle.
- 2.3. Mount the assembled needle and tubing onto the microinjection pump [1].
 - 2.3.1. Talent mounting the assembled needle and tubing onto the microinjection pump.
- 2.4. Weigh the mouse [1]. After anesthetizing the mouse via intraperitoneal injection, confirm that the toe pinch reflex ceases [2-TXT] and respiration becomes slow and steady [3]. Apply depilatory cream to the cervical region to remove hair over the lymph nodes [4]. Shave the dorsal neck skin [5]. NOTE: The VO is added for the moved shot 2.6.1
 - 2.4.1. Talent weighing the mouse.
 - 2.4.2. Close-up of the hind paw showing no response when the toe is pinched. **TXT: Anesthesia: Avertin 200 mg/kg**
 - 2.4.3. Close-up of the mouse thoracic region showing slow and steady respiration.
 - 2.4.4. Talent applying depilatory cream to the cervical region.

- 2.6.1 Talent shaving the dorsal neck skin. **NOTE: This was placed here as per author's request.**
- 2.5. Place the mouse in a stereotaxic frame on a heating pad [1]. Tilt the head at a 120-degree angle to the trunk [2].
 - 2.5.1. Talent positioning the mouse in a stereotaxic frame.
 - 2.5.2. Shot of head being tilted to 120° angle to the trunk.
- 2.6. ~~Shave the dorsal neck skin [1].~~ Clean the area sequentially with 75 percent ethanol [2].
 - ~~2.6.1. Talent shaving the dorsal neck skin.~~ **NOTE: The authors requested to move this between 2.4.4 and 2.5.1**
 - 2.6.2. Talent cleaning the area with ethanol.
- 2.7. Apply erythromycin ophthalmic ointment [1].
 - 2.7.1. Close-up of ointment being applied to the mouse eye.
- 2.8. Make a midline incision in the skin of the neck [1]. Using two pairs of blunt-tipped forceps, separate the muscle layers [2].
 - 2.8.1. Talent making a midline incision.
 - 2.8.2. Talent separating muscle layers using blunt-tipped forceps.
- 2.9. Using fine-tipped forceps, dissect through the muscle until the translucent dura mater overlying the cisterna magna or CM (C-M) becomes visible [1]. Retract the muscles to maintain consistent exposure [2].
 - 2.9.1. Talent dissecting muscle layers with fine-tipped forceps.
 - ~~2.9.2. Close-up showing SCOPE: Show the exposed translucent dura mater.~~ **NOTE: This shot is replaced with a SCOPE shot.**
- 2.10. Under a stereomicroscope, use blunt forceps to hold the needle [1]. Insert the needle at a 45-degree angle to the plane of the dura mater to a depth of approximately 1 to 2 millimeters [2]. Position the needle bevel parallel to the dura mater so that it lies completely beneath it [3]. ~~Apply gentle force with the needle to puncture the dura mater [4].~~ **Author's NOTE: Remove it**
 - 2.10.1. SCOPE: View of needle being positioned using blunt forceps.
 - 2.10.2. SCOPE: Needle being inserted at 45° angle to the plane of the dura mater.
 - 2.10.3. SCOPE: Needle bevel being positioned parallel to the dura mater.
 - ~~2.10.4. SCOPE: Shot of dura mater being punctured with the needle.~~
- 2.11. Use absorbent paper to dry any leaking cerebrospinal fluid or CSF (C-S-F) [1].
 - 2.11.1. SCOPE: Close-up of absorbent paper touching and absorbing leaking CSF.

- 2.12. Set the microinjection pump to deliver at 1 microliter per minute [1]. Inject 5 microliters of Ovalbumin, Alexa Fluor 647 Conjugate solution into the CM and leave the needle in place for 2 minutes to prevent backflow [2].
- 2.12.1. **SCREEN:** Show microinjection pump settings being adjusted to 1 $\mu\text{L}/\text{min}$. **NOTE:** Use the videographer's captured shot since the author has uploaded a still screenshot.
- 2.12.2. **SCOPE:** The solution is being injected into the CM, and the needle remains in position without movement.
- 2.13. ~~Carefully withdraw the needle [1].~~ Apply one drop of biological glue onto the meninges [2].
- 2.13.1. ~~Talent withdrawing the needle.~~ **Author's NOTE: Remove it.**
- 2.13.2. Close-up of one drop of glue being applied onto the meninges.
- 2.14. Close the surgical incision [1].
- 2.14.1. Talent suturing the incision.
- 2.15. Perform in vivo imaging of the mouse to monitor dye intensity in the cervical lymph nodes or CLN (*C-L-N*) at 30 minutes and 60 minutes [1]. In the IVIS Acquisition Control Panel, select **Imaging Wizard** and click **Next** in the pop-up window [2]. Select **Spectral Unmixing/ Filter Scan** and click **Next** [3]. Right-click on the Probes name, choose **Dyes**, select **Alexafluor 660** (*alexafluor-six-sixty*), and click **Next** [4]. Select **Manual Settings**, set the exposure time to 0.5 seconds, and use the mode ~~Use an exposure time of 0.5 seconds~~ with an excitation filter at 640 nanometers and an emission filter at 680 nanometers [5]. **NOTE:** The sentence numbers are adjusted to accommodate the added shots.
- 2.15.1. **SCREEN:** 2.15.1-Display-imaging-system-capturing-fluorescence-in-lymph-nodes.mp4: 00:00 – 00:01
 Added shot: **SCREEN:** 2.15.2-Show-imaging-parameters-being-set.mp4: 00:00-00:02
 Added shot: **SCREEN:** 2.15.2-Show-imaging-parameters-being-set.mp4: 00:03-00:05
 Added shot: **SCREEN:** 2.15.2-Show-imaging-parameters-being-set.mp4: 00:06-00:12
- 2.15.2. **SCREEN:** 2.15.2-Show-imaging-parameters-being-set.mp4: 00:13-00:28

3. Isolation of the Dorsal Aspect of the Mouse Skull

Demonstrator: Rong Wang

- 3.1. After euthanizing the mouse, perform cardiac perfusion with 20 milliliters of 1x PBS or normal saline [1-TXT].
 - 3.1.1. Talent performing cardiac perfusion. **TXT: Euthanasia: Avertin Overdose at 600 mg/kg followed by decapitation**
- 3.2. Make an incision along the midline of the skull to open the scalp [1].
 - 3.2.1. Close-up of the incision being made along the midline of the skull.
- 3.3. Using two curved hemostats, separate the scalp bilaterally by gentle retraction [1]. Insert the curved hemostats into the orbital fossae [2] and strip off the residual scalp from the nasal and oral regions [3].
 - 3.3.1. Talent retracting the scalp bilaterally using curved hemostats.
 - 3.3.2. Close-up of hemostats being inserted into the orbital fossae.
 - 3.3.3. Talent removing remaining scalp tissue from the nasal and oral regions.
- 3.4. Remove the mandible while keeping the skull intact [1].
 - 3.4.1. Close-up of the mandible being removed.
- 3.5. Remove the bone from the base of the skull to expose the ventral aspect of the brain [1].
 - 3.5.1. Talent removing bone from the skull base.
- 3.6. Using blunt forceps, carefully extract the brain while avoiding contact with the dura mater of the parietal bones [1].
 - 3.6.1. Close-up of brain extraction using blunt forceps.
- 3.7. Completely immerse the dorsal skull in 5 milliliters of 4 percent paraformaldehyde [1]. Place the sample at 4 degrees Celsius for 24 hours [2].
 - 3.7.1. Shot of skull being immersed in paraformaldehyde solution.
 - 3.7.2. Talent placing the sample in refrigerator.

4. Dissection of meninges from the mouse skull

Demonstrator: Baokai Zhang

- 4.1. Once the fixed skull reaches the room temperature, wash it with 5 milliliters of 1x PBS and repeat the wash three times for 10 minutes each [1].
 - 4.1.1. Talent adding PBS to the dish containing fixed skull.
- 4.2. Place the skull dorsal side down in a 10-centimeter dish filled with 1x PBS [1].
 - 4.2.1. Talent positioning the skull dorsal side down in the dish.

- 4.3. Using fine-tipped forceps, grasp or press down on the tympanic bulla [1] and remove it from the skull [2].
 - 4.3.1. Close-up of tympanic bulla being pressed down using fine-tipped forceps.
 - 4.3.2. Close-up of the tympanic bulla being removed from the skull. Videographer's NOTE: 4.3.1 and 4.3.2 are combined in one shot.
- 4.4. Employing scissors, trim along the ventral surface of the skull for about 2 millimeters to smooth it [1].
 - 4.4.1. Close-up of scissors trimming the ventral skull surface.
- 4.5. With forceps, clear residual white tissue from the confluence of sinuses or COS (C-O-S) region of the meninges [1].
 - 4.5.1. Close-up of residual white tissue being removed from the COS region.
- 4.6. Using fine micro forceps, gently lift one edge of the meninges from the skull margin [1]. Lift the opposite edge [2].
 - 4.6.1. Close-up of lifting one edge of the meninges.
 - 4.6.2. Close-up of lifting the opposite edge.
- 4.7. Grasp the bowl-shaped rim of the meninges [1] and pull to separate it from the skull in the COS region [2].
 - 4.7.1. Close-up of forceps holding the rim of the meninges.
 - 4.7.2. Close-up of rim of the meninges being pulled to separate it from the skull. NOTE: 4.7.1 and 4.7.2 are combined in one shot
- 4.8. Remove the maxillary bone with scissors [1].
 - 4.8.1. Talent removing the maxillary bone using scissors.
- 4.9. Grasp the rim of the meninges again [1] and pull to separate it from the skull at the frontal bone area [2].
 - 4.9.1. Close-up of meninges rim being grasped.
 - 4.9.2. Close-up of meninges rim being pulled to separate it from the skull. NOTE: 4.9.1 and 4.9.2 are combined in one shot
- 4.10. Place the intact meninges into a 24-well plate containing 1 milliliter of 1x PBS [1].
 - 4.10.1. Close-up of the meninges being placed into a well plate containing buffer.
- 4.11. After blocking and staining the meninges with primary and secondary antibodies, perform imaging [1].

4.11.1. SCREEN: 4.11.1-Display-the-fluorescence-imaging-system-capturing-the-stained-meninges.mp4: 00:00-00:05

Results

5. Results

- 5.1. In vivo imaging revealed distinct oval-shaped fluorescent signals distributed bilaterally in the cervical region following injection [1].
 - 5.1.1. LAB MEDIA: Figure 6A. *Video editor: Emphasize the two oval-shaped blue green bright spots with arrows labeled as cLNs.*
- 5.2. The tracer was clearly visible in the CLNs after injection, with fluorescence intensity peaking at approximately 60 minutes [1].
 - 5.2.1. LAB MEDIA: Figure 6B. *Video editor: Emphasize the bright spots in blue, green, red, and yellow colour with arrows labeled as cLNs.*
- 5.3. Fluorescent signals were observed in the isolated superficial CLN [1] and deep cervical lymph nodes [2].
 - 5.3.1. LAB MEDIA: Figure 6C. *Video editor: Highlight the three bright spots inside the white dashed oval labeled superficial cervical lymph nodes*
 - 5.3.2. LAB MEDIA: Figure 6C. *Video editor: Highlight the single bright spot inside the red circle labeled deep cervical lymph nodes*
- 5.4. Failed injections resulted in no detectable fluorescent signal in the cervical region [1].
 - 5.4.1. LAB MEDIA: Figure 6D. *Video editor: Circle and highlight the top center area of the image where the body is bright white.*
- 5.5. Immunofluorescence staining for lymphatic vessel endothelial hyaluronan receptor 1 or LYVE-1 (*L-Y-V-E-one*) clearly delineated the morphology of lymphatic vessels [1].
 - 5.5.1. LAB MEDIA: Figure 7A–D. *Video editor: Emphasize the bright green lines and the labels COS, TS, and SSS across the panels.*
- 5.6. A discontinuous LYVE-1 signal due to meningeal tearing was observed, compromising reliable quantification [1].
 - 5.6.1. LAB MEDIA: Figure 7E. *Video editor: Emphasize the 2 arrows, 2 green lines close to the 2 arrows, and the label Discontinuous signal.*
- 5.7. The absence of the transverse sinus region was visible in compromised samples [1].
 - 5.7.1. LAB MEDIA: Figure 7E. *Video editor: Emphasize the label Absence of the TS region, along with the white dashed oval region and the green lines inside*

Pronunciation guides:

1. Polyethylene
Pronunciation link: <https://www.merriam-webster.com/dictionary/polyethylene>
IPA: /ˌpɑːliˈeθəˌliːn/
Phonetic Spelling: paa·lee·eh·thuh·leen
2. Microinjection
Pronunciation link: <https://www.merriam-webster.com/dictionary/microinjection>
IPA: /ˌmaɪkroʊɪnˈdʒɛkʃən/
Phonetic Spelling: my·kroh·in·jek·shuhn
3. Anesthetizing
Pronunciation link: <https://www.merriam-webster.com/dictionary/anesthetize>
IPA: /əˈnesθəˌtaɪz/
Phonetic Spelling: uh·ness·thuh·tyze
4. Intraperitoneal
Pronunciation link: <https://www.merriam-webster.com/dictionary/intraperitoneal>
IPA: /ˌɪntrəˌperəˌtəˈniːəl/
Phonetic Spelling: in·truh·peh·ruh·tuh·nee·uhl
5. Depilatory
Pronunciation link: <https://www.merriam-webster.com/dictionary/depilatory>
IPA: /dɪˈpɪləˌtɔːri/
Phonetic Spelling: dih·pil·uh·tor·ee
6. Cervical
Pronunciation link: <https://www.merriam-webster.com/dictionary/cervical>
IPA: /ˈsɜːvɪkəl/
Phonetic Spelling: sur·vih·kuhl
7. Stereotaxic
Pronunciation link: <https://www.merriam-webster.com/dictionary/stereotaxic>
IPA: /ˌsteriəˈtæksɪk/
Phonetic Spelling: steh·ree·uh·tak·sik
8. Erythromycin
Pronunciation link: <https://www.merriam-webster.com/dictionary/erythromycin>
IPA: /ɪˌrɪθrəˈmaɪsɪn/
Phonetic Spelling: ih·rith·roh·my·sin
9. Ophthalmic
Pronunciation link: <https://www.merriam-webster.com/dictionary/ophthalmic>
IPA: /ɑfˈθælmɪk/
Phonetic Spelling: aaf·thal·mik
10. Translucent
Pronunciation link: <https://www.merriam-webster.com/dictionary/translucent>
IPA: /trænzˈluːsənt/
Phonetic Spelling: tranz·loo·suhnt

11. Dura mater
Pronunciation link: <https://www.merriam-webster.com/dictionary/dura%20mater>
IPA: /ˌdʊrə ˈmɑːtər/
Phonetic Spelling: duur-uh maa-tur
12. Cisterna magna
Pronunciation link: <https://www.merriam-webster.com/medical/cisterna%20magna>
IPA: /sɪˈstɜːnə ˈmæɡnə/
Phonetic Spelling: sis-tur-nuh mag-nuh
13. Stereomicroscope
Pronunciation link: <https://www.merriam-webster.com/dictionary/stereomicroscope>
IPA: /ˌsteri.oʊˈmaɪkrəˌskoʊp/
Phonetic Spelling: steh-ree-oh-my-kruh-skohp
14. Cerebrospinal
Pronunciation link: <https://www.merriam-webster.com/dictionary/cerebrospinal>
IPA: /səˈriːbrʊˈspaɪnəl/
Phonetic Spelling: suh-ree-broh-spy-nuhl
15. Ovalbumin
Pronunciation link: <https://www.merriam-webster.com/medical/ovalbumin>
IPA: /ˌoʊvəlˈbjuːmɪn/
Phonetic Spelling: oh-vuhl-byoo-min
16. Alexa Fluor
Pronunciation link: <https://www.howtopronounce.com/alexa-fluor>
IPA: /əˈlɛksə ˈflʊr/
Phonetic Spelling: uh-lek-suh floor
17. Meninges
Pronunciation link: <https://www.merriam-webster.com/dictionary/meninges>
IPA: /məˈnɪŋˌdʒiːz/
Phonetic Spelling: muh-nin-jeez
18. Fluorescence
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>
IPA: /flʊˈresəns/
Phonetic Spelling: flor-ess-uhns
19. Euthanizing
Pronunciation link: <https://www.merriam-webster.com/dictionary/euthanize>
IPA: /ˈjuːθəˌnaɪz/
Phonetic Spelling: yoo-thuh-nyze
20. Perfusion
Pronunciation link: <https://www.merriam-webster.com/dictionary/perfusion>
IPA: /pərˈfjuːʒən/
Phonetic Spelling: pur-fyoo-zuhhn

21. Hemostats

Pronunciation link: <https://www.merriam-webster.com/medical/hemostat>

IPA: /'hi:məˌstæt/

Phonetic Spelling: hee·muh·stat

22. Bilaterally

Pronunciation link: <https://www.merriam-webster.com/dictionary/bilaterally>

IPA: /baɪˈlætərəli/

Phonetic Spelling: by·lat·uh·ruh·lee

23. Orbital fossae

Pronunciation link: <https://www.howtopronounce.com/orbital-fossae>

IPA: /'ɔ:rbɪtəl 'fɑ:si:/

Phonetic Spelling: or·bih·tl faa·see

24. Parietal

Pronunciation link: <https://www.merriam-webster.com/dictionary/parietal>

IPA: /pəˈraɪətəl/

Phonetic Spelling: puh·ry·uh·tl

25. Paraformaldehyde

Pronunciation link: <https://www.merriam-webster.com/medical/paraformaldehyde>

IPA: /ˌperəfɔ:rˈmældəˌhaɪd/

Phonetic Spelling: peh·ruh·for·mal·duh·hyde

26. Tympanic bulla

Pronunciation link: <https://www.howtopronounce.com/tympanic-bulla>

IPA: /tɪmˈpænɪk ˈbʊlə/

Phonetic Spelling: tim·pan·ik bool·uh

27. Confluence

Pronunciation link: <https://www.merriam-webster.com/dictionary/confluence>

IPA: /'kɔ:nfluəns/

Phonetic Spelling: kaan·floo·uhns

28. Maxillary

Pronunciation link: <https://www.merriam-webster.com/dictionary/maxillary>

IPA: /'mæksəˌleri/

Phonetic Spelling: mak·suh·lair·ee