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Title: Cervical Lymph Duct-Cannulated Rat Model for Assessing Lymphatic Transport from the Head and Brain

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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, all done**

SCOPE: 2.12, 2.13, 2.14

Videographer: Please film all shots labeled as SCOPE using a SCOPE kit

- 2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **NO**

- 3. Filming location:** Will the filming need to take place in multiple locations?

NO – but we may move between different buildings or rooms during the filming process

- 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: 19

Number of Shots: 44

Introduction

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. **Zihan Zhu:** Our project examines how brain lymphatics relate to CNS diseases such as Alzheimer's, stroke, and neuroinflammation by identifying substances that drain from the brain.

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

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Wei Liu:** Existing methods lacked a practical, reliable in vivo approach to directly sample and quantify CNS-derived lymphatic outflow, which is enabled by this protocol.
 - 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. **Mingliang Tian:** This protocol enables direct biological sampling of brain-derived lymph, bridging indirect external imaging or resonance methods with measurable CNS outflow.
 - 1.3.1. 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?~~

- 1.4. **Zihan Zhu:** The protocol enables studying centrally administered drug clearance through lymphatic pathways, allowing measurement of drainage routes and clearance rates.
 - 1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next—~~

- 1.5. **Luojuan Hu:** Future studies can examine how CNS disease states alter lymphatic clearance and use this knowledge to optimize drugs to restore brain lymphatic drainage function for treatment.

- 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 University Animal Ethics Committee at China
Pharmaceutical University

Protocol

2. Cervical Lymph Cannulation in Rats for Continuous Lymph Collection and Quantification of Lymphatic Drug Transport

Demonstrator: Mingliang Tian, Wei Liu

- 2.1. To begin, pre-load approximately 0.7 milliliters of dilute anticoagulant solution into a 1 milliliter sterile insulin syringe with a 30-gauge needle for coating the inner surface of the polyethylene cannula [1-TXT]. Store the preloaded syringe under sterile conditions until use [2].
 - 2.1.1. Talent drawing 0.7 milliliters of the solution into a 1 milliliter insulin syringe with a 30 gauge needle. **TXT: Anticoagulant: Heparin/EDTA: 10 IU/mL**
 - 2.1.2. Talent placing the filled syringe into a sterile storage container.
- 2.2. To prepare cannulas, use tubing with 0.5-millimeter outer diameter and 0.3-millimeter inner diameter for both terminal sections, and 0.6-millimeter outer diameter and 0.5-millimeter inner diameter tubing for the middle section [2].
 - 2.2.1. Talent shows tubings with both sizes.
- 2.3. Using scissors, cut the cannula segments to lengths of 2 centimeters for the proximal portion connecting to the syringe needle [1], 40 centimeters for the middle section [2], and 8 centimeters for the distal portion intended for cannulation [3]. Assemble the cannula in a head-to-tail configuration [4].
 - 2.3.1. Talent measures ~~and cuts~~ the proximal cannula segment to a length of 2 centimeters.
 - 2.3.2. Talent measures ~~and cuts~~ the middle cannula segment to a length of 40 centimeters.
 - 2.3.3. Talent measures ~~and cuts~~ the distal cannula segment to a length of 8 centimeters.
 - 2.3.4. Talent aligns polyethylene tubing on a sterile surface and assembles the cannula in a head-to-tail configuration.
- 2.4. Then cut and bevel the distal end of the cannula assembly at an angle of approximately 45 degrees to facilitate smooth insertion into the cervical lymph duct [1].
 - 2.4.1. Talent bevels the distal end of the cannula at approximately a 45-degree angle, clearly showing the beveled tip.

- 2.5. Verify the patency of each cannula by connecting a pre-loaded syringe to the proximal end of the assembled cannula [1]. Gently release a small volume of solution to flush the cannula [2]. Ensure the solution exits smoothly from the distal end without evidence of leakage [3].
 - 2.5.1. Talent connects the syringe to the proximal end of the cannula.
 - 2.5.2. Talent gently expels a small volume of solution from syringe into the cannula.
 - 2.5.3. Shot of solution exiting smoothly from the distal end of the cannula with no leakage.
- 2.6. Next, position an anesthetized rat supine on a heated surgical pad maintained at 37 degrees Celsius [1-TXT]. Tilt the head and extend the neck using a small support pillow if necessary to increase exposure of the cervical region [2]. Gently restrain the forelimbs using rubber bands to minimize movement [3-TXT].
 - 2.6.1. Talent places the rat supine on a heated surgical pad and confirms the temperature setting. **TXT: Anesthesia: Isoflurane inhalation (0.6 L/min)**
 - 2.6.2. Talent tilts the head and extends the neck, placing a small support pillow under the neck.
 - 2.6.3. Talent gently restrains the forelimbs with rubber bands. **TXT: Secure head in anesthesia nose cone**
- 2.7. Rinse the fur over the cervical region with sterile normal saline to facilitate shaving [1]. Then thoroughly shave the entire surgical site to ensure complete removal of fur prior to incision [2].
 - 2.7.1. Talent applies sterile normal saline over the cervical fur.
 - 2.7.2. Talent shaves the cervical region completely to expose the surgical site.
- 2.8. Now mark the surgical site by drawing a 2.5-centimeter dotted line to confirm the length of the initial incision [1]. Place the upper end of the incision approximately 1 to 1.5 centimeters below the lower lip [2].
 - 2.8.1. Talent draws a 2.5 centimeter dotted line on the cervical region.
 - 2.8.2. Talent indicates the upper end of the marked line positioned below the lower lip.
- 2.9. With a sterile scalpel, carefully incise the superficial layer of the cervical musculature along the marked line [1]. Then gently separate the remaining muscle layers, primarily the superficial cervical fascia, to expose the cervical artery [2].
 - 2.9.1. Talent incises the superficial cervical musculature with a sterile scalpel.
 - 2.9.2. Talent gently separates muscle layers to expose the cervical artery.
- 2.10. Retract the sternomastoid muscle and submandibular gland [1] and tilt them away from the surgical field to facilitate identification of the cervical lymph duct [2].

2.10.1. Talent retracts the sternomastoid muscle and submandibular gland away from the surgical field.

2.10.2. Talent points to the cervical lymph duct.

2.11. Use a tissue expander to maintain the surgical window [1]. With a cotton swab, temporarily hold aside muscle tissue while locating the lymph node and associated lymph duct [2].

2.11.1. Talent positions a tissue expander to maintain the surgical window.

2.11.2. Talent uses a cotton swab to hold muscle tissue aside while searching for the lymph node and lymph duct.

2.12. Locate the opaque colorless cervical lymph duct running parallel to the carotid artery [1]. Using blunt forceps, gently pass through the separated connective tissues and maintain the forceps in position [2].

Videographer: Please film all shots labeled as SCOPE using a SCOPE kit

2.12.1. SCOPE: 2.12.1.mov 00:00-00:08

2.12.2. SCOPE: 2.12.2.mov 00:00-end

2.13. Carefully isolate the cervical lymph duct from surrounding connective and adipose tissues by blunt dissection [1]. Then create a small incision in the upper half of the exposed vessel [2].

2.13.1. SCOPE: 2.13.1.mov. 00:00-end

2.13.2. SCOPE: 2.13.2.mov. 00:00-end

2.14. Gently insert the distal end of the cannula approximately 2 millimeters into the lymph duct through the incision, orienting it toward the head [1]. Using forceps for guidance, ensure the beveled face is directed upwards [2].

2.14.1. SCOPE: 2.14.2.mov 00:10-00:34

2.14.2. SCOPE: 2.14.1.mov 00:00-end

AUTHOR'S NOTE: Move 2.14.2 before 2.14.1

2.15. Monitor the proximal end of the cannula for 1 to 2 minutes [1]. Confirm successful cannulation by observing a slow and steady outflow of lymph fluid [2]. Secure the cannula by applying a small drop of cyanoacrylate tissue adhesive with a pipette at the incision site and the secondary fixation hole [3].

2.15.1. Shot of the proximal end of the cannula.

2.15.2. Shot of steady lymph outflow from the cannula.

2.15.3. Talent applies cyanoacrylate tissue adhesive using a pipette.

2.16. Observe lymph flow for several minutes to confirm steady outflow [1]. Then carefully remove the tissue expander [2] and reposition the submandibular gland to its original

location [3].

2.16.1. Shot of continued steady lymph flow from the cannula.

AUTHOR'S NOTE: 2.15.2 and 2.16.1 were combined into a single scene (2.15.2)

2.16.2. Talent removes the tissue expander.

2.16.3. Talent repositions the submandibular gland.

2.17. Attach a pre-labelled anticoagulant-coated microcentrifuge tube to the distal end of the cannula [1]. Then reposition all muscle layers to their original locations [2] and close the skin incision with sutures and or tissue adhesive [3].

2.17.1. Talent attaches the microcentrifuge tube to the cannula.

2.17.2. Talent repositions muscle layers.

2.17.3. Talent closes the skin incision.

2.18. Collect lymph continuously into anticoagulant-coated microcentrifuge tubes, replacing the tubes at predetermined intervals, typically hourly [1]. Store the collected samples with icepacks in a cooler box [2].

2.18.1. Shot of labeled tubes with lymph.

2.18.2. Talent places collected tubes into a cooler box with icepacks.

Results

3. Results

- 3.1. Hourly lymph flow rates measured over a 6-hour collection period in successfully cannulated rats ranged from 0.09 to 0.13 milliliters per hour [1].

- 3.1.1. LAB MEDIA: Figure 7. *Video editor: Highlight the plotted data points*

- 3.2. In healthy rats, cumulative lymphatic transport of intrastriatally administered tritium-labelled albumin remained negligible during the first 2 hours [1] and increased substantially thereafter [2].

- 3.2.1. LAB MEDIA: Figure 8A. *Video editor: Highlight the all 3 cumulative transport curves from 0 to 2 hours.*

- 3.2.2. LAB MEDIA: Figure 8A. *Video editor: Highlight the portion of the 3 curves after 2 hours when it suddenly increases*

- 3.3. Comparison of tritium-labelled albumin concentrations between lymph and plasma demonstrated differing concentration profiles over time in healthy rats [1].

- 3.3.1. LAB MEDIA: Figure 8B.

- 3.4. Neuroinflammation induced by intraperitoneal administration of *Escherichia coli* lipopolysaccharide was associated with a slightly reduced overall lymph flow rate over 8 hours [1] and a nearly two-fold reduction in the proportion of total dose transported into lymph [2].

- 3.4.1. LAB MEDIA: Figure 9A *Video editor: Highlight the green lymph flow rate curve*

- 3.4.2. LAB MEDIA: Figure 9B . *Video editor: Highlight the green curve*

- 3.5. The lymph-to-plasma concentration ratio showed no significant difference between healthy and neuro-inflamed rats, indicating similar cervical lymphatic clearance profiles, [1].

- 3.5.1. LAB MEDIA: Figure 9C.

Pronunciation Guide:

Cervical

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

IPA: /'sɜː.vɪ.kəl/

Phonetic Spelling: sur·vih·kuhl

Lymph

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

IPA: /lɪmf/

Phonetic Spelling: limf

Cannulation

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

IPA: /ˌkæn.jəˈleɪ.ʃən/

Phonetic Spelling: kan·yuh·lay·shun

Lymphatic

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

IPA: /lɪmˈfæt.ɪk/

Phonetic Spelling: lim·fat·ik

Neuroinflammation

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

IPA: /ˌnʊr.oʊ.ɪn.fləˈmeɪ.ʃən/

Phonetic Spelling: nyur·oh·in·fluh·may·shun

Intrastriatal

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

IPA: /,ɪn.trə'straɪ.ə.təl/

Phonetic Spelling: in·truh·stry·uh·tuhl

Albumin

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

IPA: /æl'bjuː.mən/

Phonetic Spelling: al·byoo·min

Tritium

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

IPA: /'triː.ti.əm/

Phonetic Spelling: trih·tee·uhm

Escherichia coli

Pronunciation link: <https://www.merriam-webster.com/dictionary/Escherichia%20coli>

IPA: /ɛʃ.ə'rɪk.i.ə 'koʊ.laɪ/

Phonetic Spelling: esh·uh·rik·ee·uh koh·ly

Lipopolysaccharide

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

IPA: /,lɪp.oʊ.pɑː.li'sæk.ə.raɪd/

Phonetic Spelling: lih·poh·paa·lee·sak·uh·ryd

Submandibular

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

IPA: /,sʌb.mæn'dɪb.jə.lə/

Phonetic Spelling: sub·man·dib·yuh·lur

Sternomastoid

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

IPA: /,stɜː.noʊ'mæs.tɔɪd/

Phonetic Spelling: stur·noh·mas·toyd

Cyanoacrylate

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

IPA: /saɪ.æn.oʊ'æk.rə.leɪt/

Phonetic Spelling: sy·an·oh·ak·ruh·layt

Polyethylene

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

IPA: /,pɑː.li'εθ.ə.li:n/

Phonetic Spelling: pah·lee·eth·uh·leen

Isoflurane

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

IPA: /,aɪ.sou'flʊr.eɪn/

Phonetic Spelling: eye·soh·floo·rayn

Cannula

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

IPA: /'kæn.jə.lə/

Phonetic Spelling: kan·yuh·luh

Anticoagulant

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

IPA: /,æn.ti.koʊ'æg.jə.lənt/

Phonetic Spelling: an·tee·koh·ag·yuh·luhnt

Microcentrifuge

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

IPA: /,maɪ.kroʊ'sɛn.trɪ.fjuːdʒ/

Phonetic Spelling: my·kroh·sen·trih·fyooj

Heparin

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

IPA: /'hep.ə.rɪn/

Phonetic Spelling: hep·uh·rin

Adipose

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

IPA: /'æd.ɪ.pəʊs/

Phonetic Spelling: a·duh·pohs