

PROTOCOL:

The procedure(s) described in this protocol were approved by the university animal ethics committee and were performed in accordance with the experimental ethical guidelines of China Pharmaceutical University. Prior to commencing any animal-related procedures, obtain the necessary approvals from the relevant institutional or organizational ethics committee. Male Sprague-Dawley (SD) rats weighing 220 to 250 g are used as the experimental animals. Animals are sourced from authorized experimental animal suppliers (refer to **Table of Materials** for details of the licensed experimental animal providers) and housed in dedicated cages with free access to food and water until the commencement of the experiment. In this protocol, cannulation of the left deep cervical lymph duct is described as a representative example. Currently, there is limited evidence describing anatomical differences between the left and right deep cervical lymph ducts. Based on observations from previously completed experiments, no significant differences have been noted between the two sides, including in lymph flow rates.

1. Safety precautions and procedures

1.1 Perform all procedures in accordance with institutional biosafety guidelines, and all personnel should receive adequate training in microsurgical techniques, experimental animal handling, and chemical safety prior to conducting this protocol.

1.2 Wear appropriate personal protective equipment (PPE), including a laboratory coat, surgical gloves, and protective goggles, throughout the procedure. As a volatile anesthetic (isoflurane) is used and may cause central nervous system depression and cardiovascular toxicity in humans following prolonged exposure, perform the procedure in a well-ventilated environment. Food and drink must not be brought into the laboratory and ensure that flowing water is readily accessible at all times.

1.3 Handle all sharp items, including syringe needles, razor blades, and micro-scissors, with care and dispose of them immediately after use in designated sharps containers; under no circumstances should disposable sharp items be reused.

1.4 Treat all biological samples (e.g., collected lymph) and animal body fluids (e.g., blood) as potentially hazardous, and handle all collection equipment carefully while keeping samples away from the surgical area. In the event of an accidental needle-stick injury or skin contact with hazardous substances, immediately wash the affected area with soap and water and report the incident in accordance with institutional safety protocols.

2. Preparation on the day before the surgery

2.1 Fast or feed the rats according to the requirements of the specific experiment. This protocol does not mandate a specific fasting or fed state.

2.2 Prepare an anticoagulant solution by diluting heparin or ethylenediaminetetraacetic acid (EDTA) to a final concentration of 10 IU/mL. Pre-load this mixture (approximately 0.7 mL) into a 1 mL sterile insulin syringe with a 30 G needle, to be used to coat the inner surface of the polyethylene (PE) cannula (more information discussed in step 1.3). Store this prepared mixture under sterile conditions until use.

2.3 Prepare cannulas for lymph and blood sample collection by assembling a PE cannula in a head-to-tail configuration, using 0.5 mm outer diameter (OD), 0.3 mm inner diameter (ID) tubing for both terminal sections and 0.6 mm OD, 0.5 mm ID tubing for the middle section.

2.3.1 Cut the cannula segments to lengths of 2 cm for the proximal portion connecting to the syringe needle, 40 cm for the middle section, and 8 cm for the distal portion intended for cannulation.

2.3.2 Cut and bevel the distal end of the cannula assembly at an angle of approximately 45° in order to facilitate smooth insertion into the cervical lymph duct. Refer to **Figure 2** for a detailed illustration of the cannula assembly.

[Place FIGURE 2 Here]

NOTE: This bevel facilitates smooth insertion into the lymph duct and reduces the risk of blockage by increasing the effective cross-sectional area. Following successful cannulation, the proximal (2 cm) section is disconnected, and lymph is collected directly from the exposed proximal end of the middle section (this is discussed in further detail in step 3.9).

2.4 Prepare the anesthesia inhalation system by pre-loading liquid isoflurane into the pressurized container equipped with a vaporizer. Prior to use, perform a test run (priming) of the vaporizer to confirm airtightness and to ensure stable and consistent gas flow.

2.5 If required, prepare the test drug candidate in a suitable vehicle. Conduct stability studies to confirm formulation integrity during storage and throughout the administration period. Include additional agents (e.g., biological dyes such as Evans Blue) where applicable.

2.6 Prepare the containers for lymph collection by pre-weighing sterile microcentrifuge tubes and recording their initial weights. Label each tube sequentially according to the planned collection time points (e.g., hourly intervals). Add concentrated heparin (or EDTA) to each tube and remove the solvent by nitrogen blowdown evaporation, leaving 2 to 5 IU of dried heparin (or EDTA) per tube to prevent uneven dilution of collected lymph.

3. Preparations immediately prior to commencing the surgery

3.1 Verify the patency of each cannula by flushing with the prepared anticoagulant solution. Connect a syringe to the proximal end of the assembled cannula and gently release a small volume

of solution. Ensure that the solution exits smoothly from the distal end without evidence of leakage. If leakage is suspected, as typically indicated by fluid escaping at the connection point(s), discard the entire cannula assembly and reconstruct it according to step 1.3.

NOTE: This step simultaneously pre-coats the inner surface of the cannula with anticoagulant (this step may also be conducted the day before surgery, so the cannula is precoated overnight).

3.2 Start administering inhaled isoflurane through a nose-cone mask approximately 5 min prior to the commencement of surgery. Maintain anesthesia throughout the procedure at a delivery rate of 0.6 L/min under a pressure of 101.3 kPa at 20 °C. Ensure depth of anesthesia using the toe pinch response.

3.2.1 Continuously monitor the depth of anesthesia. Immediately euthanize the rat if signs of distress or physiological instability are observed.

3.3 Position the rat supine on a heated surgical pad maintained at 37 °C. Tilt the head and extend the neck, using a small support (pillow) if necessary, to increase exposure of the cervical region. Secure the head within the anesthesia mask to ensure continuous delivery of isoflurane and gently restrain the forelimbs using rubber bands to minimize movement during the procedure.

3.4 Rinse the fur over the cervical region with sterile normal saline to facilitate shaving. Thoroughly shave the entire surgical site (using a double-edged safety razor blade), typically the neck and clavicle region, to ensure complete removal of fur prior to incision (refer to **Figure 3A** for an example of the shaved neck area).

3.5 Clean the entire shaved area with disinfectants, such as an iodine-containing agent.

3.6 Mark the surgical site by drawing a 2.5 cm dotted line to confirm the length of the initial incision. Position the line slightly to the right of the midline of the rat's body. Place the upper end of the incision approximately 1 to 1.5 cm below the lower lip (refer to **Figure 3B** for an example of the incision site, with measurements aided by a ruler).

[Place **FIGURE 3** Here]

4. Cannulation of the cervical lymph duct

4.1 Position the rat with its head pointed towards the operator. Use a microscope for this step due to the relatively small diameter of the cervical lymph duct.

4.2 Using a sterile scalpel, carefully incise the superficial layer of the cervical musculature along the aforementioned 2.5 cm-long mark, oriented nearly parallel to the midline (refer to **Figure 4A** for the opened superficial skin layer).

4.3 Gently separate the remaining muscle layers, primarily the superficial cervical fascia, to expose the cervical artery, which is typically distinguishable by its red coloration.

4.4 Retract the sternomastoid muscle and submandibular gland and carefully tilt them away from the surgical field to facilitate identification of the cervical lymph duct. Employ a tissue expander to maintain the surgical window and use a cotton swab to temporarily hold aside the muscle tissue while locating the lymph node and associated lymph duct. Ensure the tissue expander is in place throughout the entire cannulation process (refer to **Figure 4B** for the opened surgical window with the tissue expander in place).

4.5 Locate the cervical lymph duct (preferably under a microscope): The duct appears as an opaque, colorless vessel with approximately 0.2 to 0.5 mm in diameter and runs immediately parallel to the carotid artery. One or more adjacent, flesh-colored dCLNs can often be observed along the course of the cervical lymph duct (refer to **Figure 4C** for a microscopic view of the exposed deep cervical lymph duct and an adjacent dCLN).

NOTE: The cervical lymph ducts typically have a color very similar to the surrounding tissues, and their identification often requires careful inspection, particularly in rats with more fat tissue. For surgical practice purposes (but not for studies on lymphatic transport of drugs or endogenous substances), Evans Blue may be subcutaneously injected under the neck at the beginning of the surgery so that the cervical lymph duct can be better visualized when the dye drains via the lymphatics.

4.6 Using a pair of blunt forceps, gently pass through the separated connective tissues and maintain the forceps in position to ensure the cervical lymph duct remains clearly visible within the surgical field.

4.7 Carefully isolate the cervical lymph duct from the surrounding connective and adipose tissues by blunt dissection, taking particular care to avoid damage, as the vessel is extremely fragile.

4.8 Use a pair of micro scissors to create a small incision in the upper half of the exposed vessel. Do not detach the duct completely; the two halves must remain intact.

4.9 Prepare the cannula (as described in Step 1.3) by ensuring it is pre-filled with diluted anticoagulant solution using an insulin syringe attached to a 30 G needle, with no air bubbles present. Gently insert the distal end of the cannula approximately 2 mm into the lymph duct through the incision, orienting it towards the head. Use forceps for guidance, ensuring the beveled face is directed upwards (refer to **Figure 4D** for a successful cannulation of the cervical lymph duct). The actual depth of insertion may vary depending on the individual rat.

NOTE: Since the cannula insertion is relatively shallow in this procedure, an additional fixation point to the surrounding area can be helpful. This is achieved by creating a second hole approximately 1.5 cm from the surgical window using scissors, carefully penetrating through the skin (Additional details are shown in **Figure 4E** when tissue adhesives are used). The cannula should be passed through the hole to assist in securing its position. This opening will be further stabilized in subsequent steps.

4.10 Detach the insulin syringe together with the proximal section of the cannula assembly. Monitor the exit end of the collecting portion (i.e., the proximal end) of the cannula for 1 to 2 min. Confirm successful cannulation by a visible, slow, and steady outflow of lymph fluid from the cannulated duct.

4.11 Once cannulation is verified to be successful, secure the cannula by applying a small drop of cyanoacrylate tissue adhesive with a pipette at both the lymph duct incision site and the secondary fixation hole (refer to **Figure 4E** for a demonstration of cannula fixation). Take extra care to avoid occluding the vessel or impairing lymph flow.

4.12 Observe the lymph flow for several minutes to confirm a steady outflow (a visible droplet typically forms at the end of the cannula within a minute). Once verified, carefully remove the tissue expander from the surgical window and reposition the submandibular gland to its original location.

4.13 Attach a pre-labelled anticoagulant-coated microcentrifuge tube (see step 1.6) to the distal end of the cannula to collect the free-flowing lymph.

[Place **FIGURE 4** Here]

5. Post-surgical period and drug administration

5.1 Upon completion of cervical lymph duct cannulation, reposition all muscle layers to their original locations. Subsequently, close the skin incision with sutures and/or tissue adhesive; typically, a 2.5 cm long incision can be properly closed with 8 stitches (refer to **Figure 5A** for a surgical window closed with suture). Remove the constraining rubber band(s) on the forelimbs upon completion of this step.

5.2 Administer drugs into the CSF using an appropriate route, such as intra-cisterna magna (ICM), intra-cerebroventricular (ICV), or lumbar intrathecal (LIT) injection. Using ICM administration as an example, identify the injection site at the cisterna magna, located at the base of the skull and visible as the only soft depression on the dorsal surface. Shave the surrounding fur as required to facilitate visualization (refer to **Figure 5B** for a marked suitable area for ICM injection).

NOTE: To maintain intracranial pressure (ICP) during ICM injection, an equivalent volume of CSF is often withdrawn prior to drug administration. It is recommended to use a new, pre-filled syringe for injection. Bending the syringe needle can facilitate easier administration (refer to **Figure 5C** for a demonstration of ICM injection using a bent syringe needle).

5.3 Keep the rat supine on the heated surgical pad (maintained at 37 °C) to preserve body temperature after injection, and throughout the sample collection period.

[Place **FIGURE 5** Here]

6. Collection of lymph samples and analysis

6.1 Collect lymph continuously into anticoagulant-coated microcentrifuge tubes, replacing the tubes at pre-determined intervals (typically hourly). Store the collected samples with icepacks using a cooler box. Refer to **Figure 6** for a collected cervical lymph sample in a microcentrifuge tube.

6.2 Upon completion of the lymph collection process, euthanize the rat humanely according to appropriate protocols.

6.3 Determine lymph flow rate by measuring the volume of lymph collected during each planned interval (hourly).

6.4 Measure the concentrations of drugs (or substances of interest) in the lymph using appropriate analytical methods, including HPLC, HPLC-MS, or commercially available assay kits, to evaluate absorption efficiency, lymph transport rate, and related parameters.

6.5 Calculate lymphatic drug mass transport by multiplying the measured drug concentrations by the corresponding lymph volumes.

[Place FIGURE 6 Here]

7. Post-procedure handling and waste disposal

7.1 Temporarily store euthanized rat(s) in designated medical waste containers within a dedicated cold storage area (e.g., a refrigerator) until they are collected and disposed of by a professional medical waste processing team.

7.2 Collect all disposed sharp items in a designated sharps container, separate from all other waste.

7.3 Dispose of all other non-sharp items used in the experiment (e.g., cotton swabs, rubber bands, PE cannulas) in medical waste bags, and do not dispose of them with household or general waste. Ensure that no sharp items are placed in this bag.

7.4 Clean and disinfect all remaining reusable tools in accordance with laboratory protocols.