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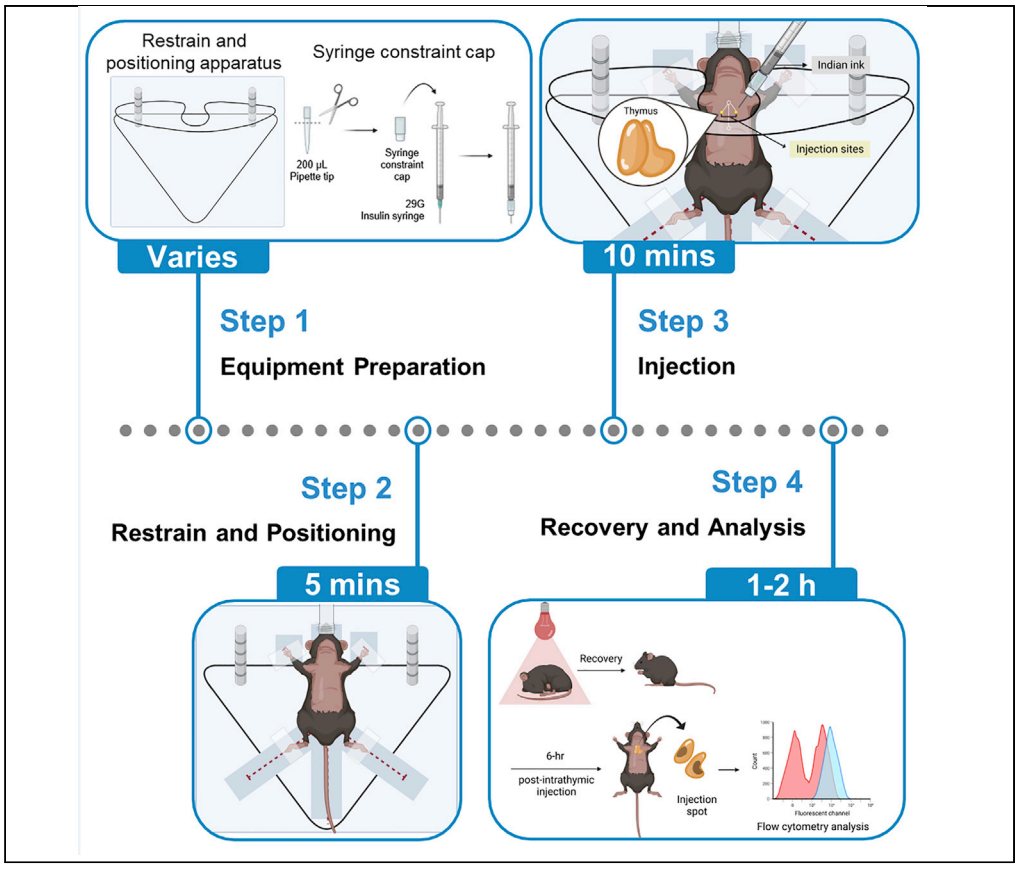
Protocol

Protocol for standardized intrathymic injection in mice



Currently available intrathymic injection techniques cause postoperative complications or difficulties in equipment acquisition. Here, we describe a standardized intrathymic injection protocol that requires only basic equipment with a minimally invasive procedure. We detail steps to identify injection sites for intrathymic delivery. We then describe how to visualize a successful intrathymic injection by including Indian ink in the injected solution.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

A standardized intrathymic injection protocol with minimally invasive procedures

The equipment required for the procedures is easily accessible

Incorporation of Indian ink allows clear visualization of intrathymic injection

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Protocol for standardized intrathymic injection in mice

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SUMMARY

Currently available intrathymic injection techniques cause postoperative complications or difficulties in equipment acquisition. Here, we describe a standardized intrathymic injection protocol that requires only basic equipment with a minimally invasive procedure. We detail steps to identify injection sites for intrathymic delivery. We then describe how to visualize a successful intrathymic injection by including Indian ink in the injected solution.

For complete details on the use and execution of this protocol, please refer to Tsai et al. (2022).[1]

BEFORE YOU BEGIN

T lymphocytes are the key arsenal of cellular immunity. They are generated and educated in the primary lymphoid organ, the thymus. To better understand T cell development and immune responses, the thymus has been a major target for many immunological studies.[2] The thymus is separated from the circulation to prevent the contact of unselected developing thymocytes with the peripheral tissue antigens.[3] This feature makes the thymus much less accessible for manipulation or drug administration via the intravenous or intraperitoneal routes. The conventional intrathymic injection involves an incision of the chest or openingof the thoracic cavity to expose the thymus that causes distress to the animal and postoperative complications.[4,5] The ultrasound-guided intrathymic injection has been successfully developed to avoid surgical procedures[6,7]; however, the proper transducer-equipped ultrasound scanner is a specialized instrument that may not be available to many researchers. Here, we describe a refined intrathymic injection protocol with standardized steps and easily accessible tools.

Institutional permissions

All mice were bred and maintained in the specific-pathogen-free room at NYCU Animal Center. All experimental procedures were approved (IACUC No. 1110804) and performed according to the NYCU Institutional Animal Care and Use Committee guidelines.

Mice

5 to 6-week-old C57BL/6 female and male mice are used in this protocol.

⚠ CRITICAL: This protocol is best optimized for 5 to 6-week-old mice. The major consideration to use 5 to 6-week-old mice is because the cellularity of the mouse thymus reaches a plateau at this age and gradually involutes afterwards. It is not recommended to use younger or older mice for intrathymic injection as the 3 to 4-week-old mice are smaller in body size and more difficult to handle and the older 7 to 8-week-old mice already

have smaller thymus. Although we expect different strains of mice at the same age to be similar in the body size and the anatomical location of thymus, the researcher needs to set out pilot experiments to determine if any minor adjustments are needed.

Reagent preparation

Timing: 10 min

This protocol describes the standardized intrathymic injection procedure. To mark the injection site, minute amount of diluted Indian ink is used.

1. To assemble the anesthesia apparatus, soak a regular size cotton ball (~20 mm in diameter) with 5% (v/v) of isoflurane in propylene glycol.
   a. Keep in a 15 mL tube with cap securely tightened until use.

   Note: This is an easy-made anesthesia nose cone sufficient to induce and maintain the anesthesia during the intrathymic procedure. Alternatively, if available, a gas anesthesia instrument set to deliver 1% isoflurane in oxygen at the flow rate of 450 mL/min can also be used.

   Note: The use of isoflurane inhalant anesthesia for the intrathymic injection procedure is recommended, due to its wide safety margin, and rapid return to consciousness after exposure has ended. The rapid recovery time is important – the mice that have received successful injection will be able to move around freely soon after the isoflurane nose cone is removed. Any mice that show signs of physical distress such as bleeding or difficulty in breathing should be euthanized immediately.

2. To prepare the injection solution, warm the sterilized phosphate-buffered saline (PBS) to 37°C, and dilute the Indian ink with PBS to 0.4% (v/v).
   a. Keep the prepared injection solution warm at 37°C until use.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Indian ink	Royal Talens	Cat#44727000
Propidium iodide	Sigma-Aldrich	Cat#P4864
Attane (isoflurane)	Panion & BF Biotech Inc.	Cat#4900-2104
1,2-Propanediol (1,2-PDO, propylene glycol)	Sigma-Aldrich	Cat #82280-250ML
Experimental models: Organisms/strains		
Mouse: C57BL/6 (5–6 weeks old, male/female)	The Jackson Laboratory	RRID: IMSR_JAX:000664
Software and algorithms		
FlowJo (V10)	BD	https://www.flowjo.com/solutions/flowjo; RRID: SCR_008520
Other		
29G Insulin Syringe	BD	Cat#305930
200 µL universal fit pipette tips	Corning Incorporated	Cat#4844
Clear acrylic restraint and positioning apparatus	Dr. Hong-Ren Jiang or a local supplier	http://scimaker.blogspot.com https://www.acmeplastics.com/cut-to-size-plastic
Animal clipper	Li-Fong Pet supply	Turbo 1000

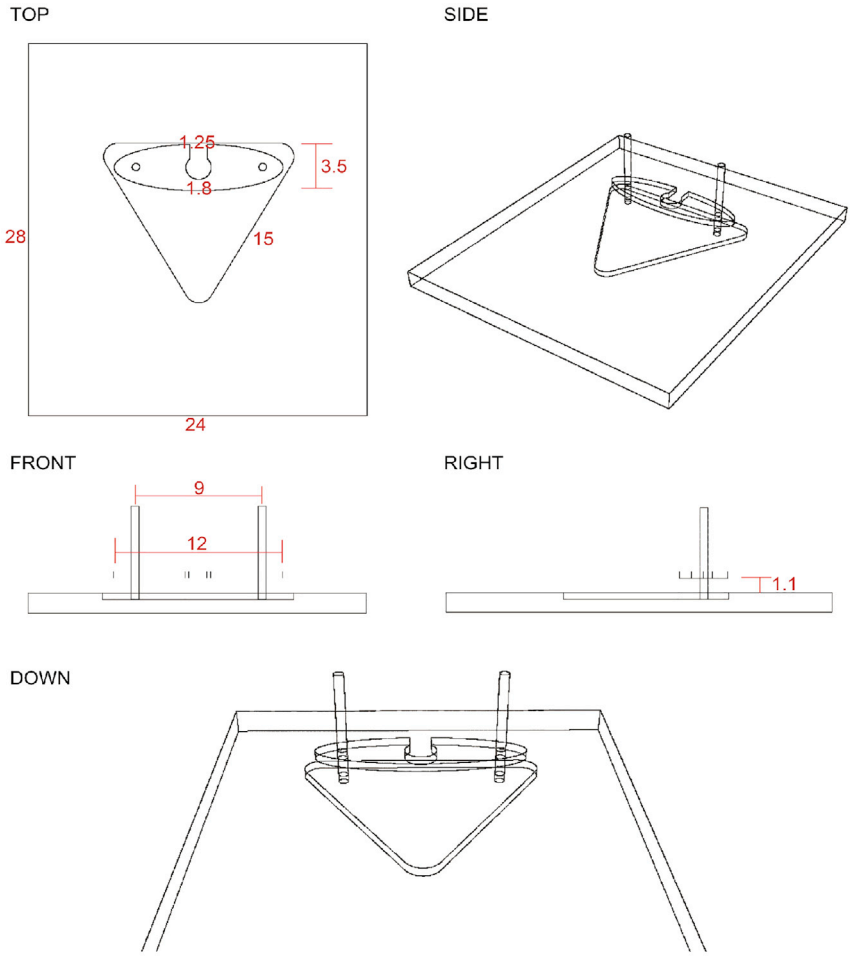

Figure 1. The specifications of the acrylic restraint and positioning apparatus

The intrathymic injection working station is made with two clear acrylic plates bolted to a Styrofoam base. The users can modify the size and shape of the acrylic plates to best fit their operation.

MATERIALS AND EQUIPMENT

Clear acrylic restraint and positioning apparatus

The restrain and positioning apparatus comprises a top restraint plate and a bottom positioning and stabilizing plate (See Figure 1). The apparatus is bolted to a Styrofoam working station to provide stability during the procedure.

> *Note:* The operation platform can be made by ordering from local provider (eg. https://www.acmeplastics.com/cut-to-size-plastic) with the detailed dimensions illustrated in the figure. The material of the device is not limited to acrylic but we do recommend it to be light-weighted and transparent for easy visualization. The purpose of this operation platform is to standardize the placement of mouse and to stabilize the chest during the injection.

Syringe constraint cap

The syringe constraint cap is a homemade device created by cutting the tip off from a 200 μL pipette tip. The edges are smoothened with sandpaper. The finished syringe constraint cap measures up to 150 mm in length and exposes 4.5 mm of the needle when attached to the syringe (Figure 2).

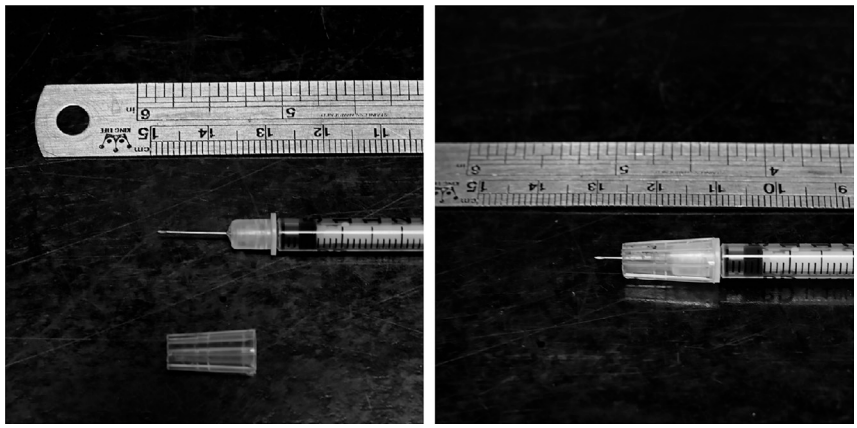

Figure 2. The syringe constraint cap

The syringe constraint cap attached to a 29G insulin syringe is shown here. Minor adjustment of the syringe constraint cap may be required if a different type of syringe is used.

STEP-BY-STEP METHOD DETAILS

Restrain and positioning of the mouse

Timing: 5 min (for step 1)

This section describes how to set up and position the mouse for injection.

1. To remove the fur of the mouse and expose the injection area.
   a. Put the mouse under anesthesia by gently covering its nose and mouth with the nose cone containing the 5% isoflurane-soaked cotton ball.
   b. Confirm the mouse is unconscious and does not move in response to stimulus (e.g., gentle pulling of the limbs).
   c. Remove the fur on the chest with a hair clipper to expose the skin.
2. To restrain and position the mouse for the injection procedure.
   a. A thin layer of ophthalmic ointment (or Vaseline) is applied to the eyes of the mouse to keep them moist.
   b. A heat lamp is set up next to the working station to ensure constant body temperature during the procedure.
   c. Place the mouse onto the restraint and positioning apparatus, and secure the mouse extremities with surgical adhesive tape on the positioning plate (Figure 3).

   Note: It is recommended to mark the positioning apparatus with a straight line – it helps to align the mouse from nose to tail and avoid tilted positioning (Figures 3A and 3B).

3. Continuous anesthesia during the procedure is provided by keeping the nose cone containing the 5% isoflurane-soaked cotton ball on the mouse.

   Note: If a gas anesthesia instrument is available, administer 1% isoflurane mixed with oxygen at the flow rate of 450 mL/min.

4. Disinfect the mouse chest with 70% isopropyl alcohol.

Injection

Timing: 10 min (for step 5)

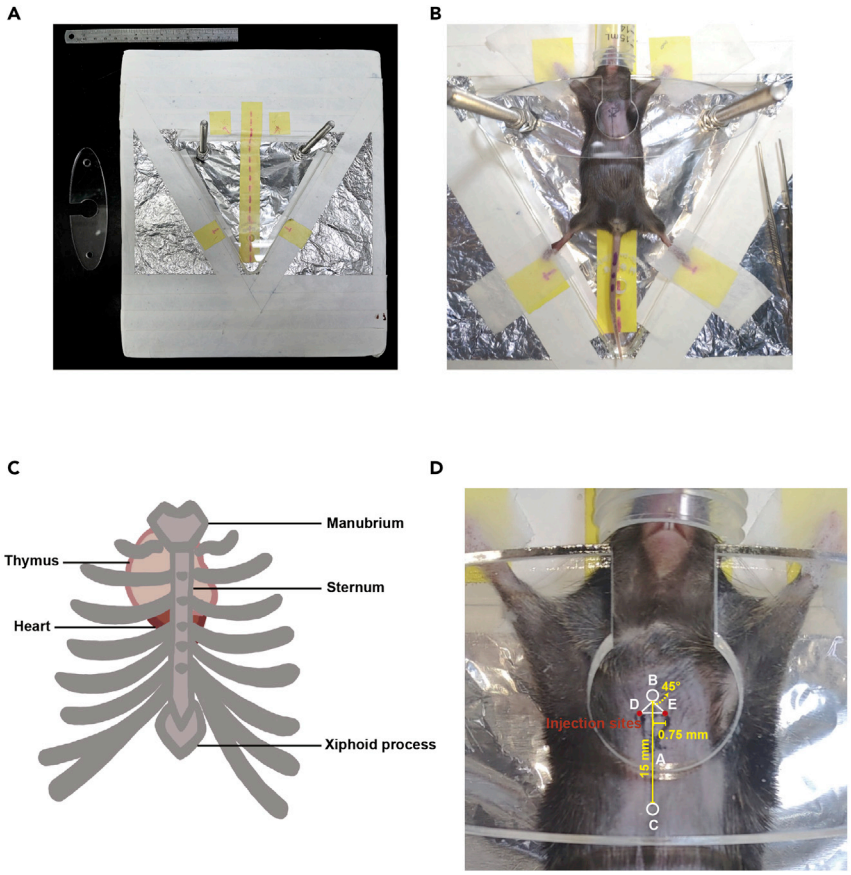

Figure 3. Instructions on mouse placement and the identification of the intrathymic injection sites

(A) The set-up of the intrathymic injection station.

(B) The mouse is aligned and secured between the restraint and positioning apparatus.

(C) The illustration of mouse chest anatomy showing the location of the thymus, the heart, the manubrium, the sternum, and the xiphoid process.

(D) The reference points and lines are marked to locate the correct injection sites.

Here we describe how to draw the reference lines to identify the correction injection sites and perform the injection.

5. To draw the reference lines with a marker on the mouse chest to identify the injection sites.

   Note: The injection sites are located in the first intercostal space on both sides of the sternum. The relative position of the sternum and the thymus are shown in Figure 3C.

   a. Find the sternum by gently touching the mouse's chest with the fingers, then draw the central line in the middle of the sternum (Figure 3D, line A).
   b. Locate the manubrium and draw a circle to label the upper edge of the chest (Figure 3D, circle B).
   c. Locate the xiphoid process and draw a circle to label the bottom of the chest (Figure 3D, circle C). The manubrium (circle B) is approximately 15 mm away from the xiphoid process (circle C).
   d. Use circle B as a reference point, and draw the extension lines at 45° angle at both left and right sides of the chest (Figure 3D, line D and E).
   e. Take a ruler and move perpendicularly along line A. The injection sites are estimated to be 0.75 $\pm$ 0.25 mm away from line A at the intersection of line D and E, respectively.

Note: We do not recommend using younger or older than 5-6 weeks-old mice for intrathymic injection. The mouse thymus reaches a growth plateau at 5–6 weeks old and will start undergoing involution thereafter. The parameters provided in this protocol are optimized for this age of mice specifically.

6. Place the restraint plate over the mouse's chest to provide a mild restriction of chest movement and create a stable injection platform.

   Note: The acrylic plates only aim to provide minimal restriction of chest movement. The plate should be supported by the nuts installed on the bolt instead of directly compressing the mouse chest. Ensure it is not pressing down too hard to affect normal mouse breathing.

7. Load 15 μL of the injection solution to the 29G BD insulin syringe.

   Note: Avoid bubbles in the syringe. The maximum injected volume allowed is 20 μL. In our experience, injection with a volume larger than 20 μL often results in tissue damage and blood clotting in the thymus.

8. Install the constraint cap on the syringe to help determine the depth of the injection.
9. Position the needle perpendicularly to the injection site, and push the needle in until the constraint cap touches the chest.

   Note: A new needle should be used for every injection of each mouse.

   Note: The intrathymic injection method published by *de la Cueva T.* et al. requires an incision of the skin and uses an injection angle of 30° with a specific depth of tissue penetration by reference to the length of the needle's bevel. In practice, we find it rather difficult to achieve – the operator needs to steadily remain the desirable injection angle while making sure the needle does not go too shallow or deep into the tissue. The technique we describe here avoids these obstacles by applying the injection perpendicularly to the mouse chest with the defined penetration depth achieved by the constraint cap.

   ⚠ CRITICAL: The needle should not be pushed further as soon as the constraint cap touches the mouse's chest. Just touching the mouse's chest with the constraint cap helps to deliver the tip of the needle at the proper depth into the thymic tissue.

10. Slowly push the plunger to deliver the injection solution into the thymic lobe, wait for 5 s, then gently withdraw the needle (please see Methods video S1: Intrathymic injection).

    ⚠ CRITICAL: If clear traces of blood are observed when withdrawing the needle, the injection has likely failed and punctured the heart. The mouse should be immediately euthanized by carbon dioxide (CO_2) inhalation.

11. Repeat steps 7–10 to inject the second thymic lobe.

Recovery and analysis

Timing: 30 min to 1 h (for step 12)

Post-injection care and exemplary analysis are provided in this section.

12. Remove the nose cone from the mouse to end the exposure to isoflurane.
    a. Keep the mouse under the heat lamp and allow it to recover from anesthesia.

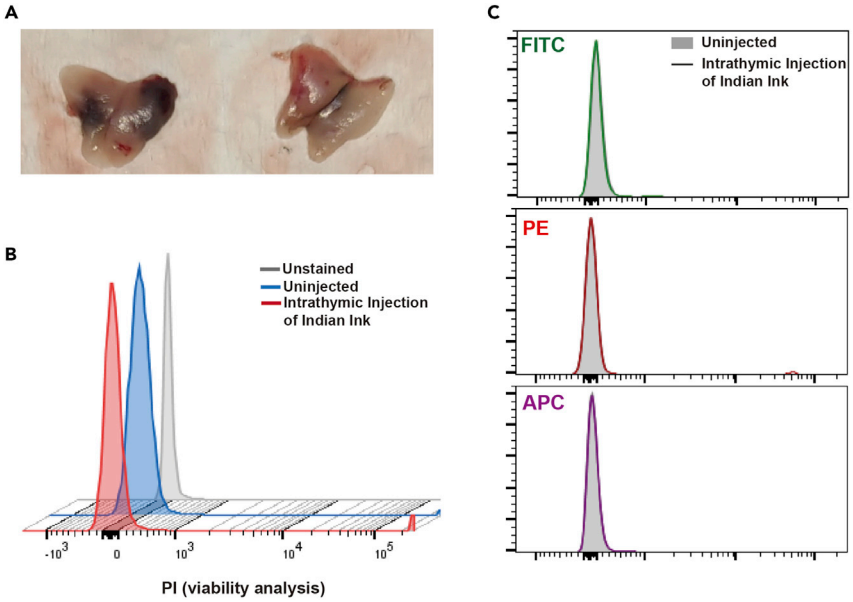

Figure 4. Post-intrathymic injection assessments

(A) The injectant (diluted Indian ink) is visible and contained within the injected thymus – left: anterior view; right: posterior view.

(B) The viability is evaluated via PI staining. Comparison among unstained, uninjected, and intrathymic injected groups show no detectable impact on cell viability for the Indian ink intrathymic injected sample.

(C) The presence of Indian ink does not interfere with fluorescence, such as FITC, PE, or APC.

b. Closely monitor the behavior of the injected mouse for at least 1 h.

Note: Mice should be euthanized when showing signs of distress or pain, including weakness, abnormal respiration rate, continuous trembling, or lethargy.

13. Depending on the experimental design, euthanize the mouse by carbon dioxide inhalation and harvest the thymus for further analysis at the desirable time points.

Note: The Indian ink can clearly label the injection spot in the thymus tissue for at least five days. Nevertheless, the injection spot becomes smaller 5 days post-injection, suggesting the longer incubation time may result in the diffusion of the dye. Therefore, we suggest using the Indian ink as a reference for the intrathymic injection only if the tissue is harvested within 5 days. Injections performed more than 5 days before the thymus harvest cannot be visually verified.

14. The thymus is harvested 6 h post-intrathymic injection and is subjected to flow cytometric analysis to determine the overall impact of the procedure.
    a. The single cell suspension is prepared, washed with PBS, and re-suspended in FACS buffer (PBS with 0.5% bovine serum albumin (BSA) and 2 mM EDTA) containing propidium iodide for viability analysis.

EXPECTED OUTCOMES

Successfully intrathymic-injected mice should show no signs of pain. An experienced operator can achievea successful injection rate between 60 to 90%. Upon tissue harvest, the injected Indian ink should be clearly visible in the thymus for 24 h (Figure 4A) to five days and does not affect cell viability (Figure 4B). As Indian ink is essentially inert fine carbon particles, it does not interfere with further fluorescent analysis, as no signal is detected in FITC-, PE-, or APC-channels as examined

via flow cytometric analysis (Figure 4C). Thus, the researchers might examine the effect of the injected compounds by their preferred methods such as flow cytometry or immunofluorescence, and compare them with vehicle-injected controls. For example, injection of the pentose phosphate pathway inhibitor 6-Aminonicotinamide (6-AN) reduced the phagocytosis of apoptotic thymocytes by thymic macrophages demonstrating the importance of this pathway for efferocytosis.[1]

LIMITATIONS

Though this intrathymic injection protocol is non-invasive and generates minimum stress to the mouse, it requires a basic understanding of mouse anatomy and practice to master the procedures.

TROUBLESHOOTING

Problem 1

Unable to locate the thymus.

Potential solution

We suggest practicing the intrathymic injection on euthanized mice first. It is crucial to get familiar with the location of the thymus and the pressure needed to insert the needle into the thoracic cavity.

Problem 2

Blood clotting.

Potential solution

Blood clotting can be observed after injection. Pushing the plunger too quickly can cause injury to the thymus structure or even the blood vessel. Maintaining the stability of the syringe during the injection prevents injury to the thymus.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and fulfilled by the lead contact, Chia-Lin Hsu (clhsu@nycu.edu.tw).

Materials availability

This study did not generate any new unique reagents.

Data and code availability

This study did not generate datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.102010.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

T.-L.T. and P.-Y.T. developed and performed the experiments. I.L.D. and C.-L.H. conceptualized the study and designed and supervised the experiments. T.-L.T., P.-Y.T., and C.-L.H. analyzed and interpreted the data and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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