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Title: Microscopic Observation of Lymphocyte Dynamics in Rat Peyer's Patches

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **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 (through a camera port or one of the oculars). Please list the make and model of your microscope.

   PENTAX PRISMVUE NF3

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**

Current Protocol Length

Number of Steps: 19
Number of Shots: 35

Introduction

1. Introductory Interview Statements

REQUIRED:

1. **Kazuhiko Shirakabe:** This protocol makes it possible to perform *in vivo* observation of lymphocyte dynamics in rat Peyer's patches for long periods of time. It can be used to explore the effects of various drugs on lymphocytes *in vivo*.

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

Introduction of Demonstrator:

2. **Kazuhiko Shirakabe:** Demonstrating the procedure will be Kazuki Horiuchi, a medical doctor from my laboratory.

    2.1. INTERVIEW: Author saying the above.

    2.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

Ethics Title Card

3. Procedures involving animal subjects have been approved by the Animal Research Committee of National Defense Medical College.

Protocol

2. Collection and Separation of Lymphocytes

2.1. Begin by forming a cannulation tube **[1-TXT]** and dipping it in hot water **[2]**, then bend and fix it to a plastic board with adhesive tape **[3]**.

2.1.1. Talent forming a cannulation tube. **TEXT: 1 mm diameter**

2.1.2. Talent dipping the tube in hot water.

2.1.3. Talent fixing the tube to a plastic board.

2.1.4. ~~Talent drawing a curve.~~

2.2. After anesthetizing a male Wistar rat and removing the hair on its abdomen, make a horizontal incision with surgical scissors from the midline to the left subcostal area **[1]**, taking care not to damage the blood vessels in the parietal peritoneum **[1A]**. NOTE: the scope was actually magnifying glasses so no scope footage.

2.2.1. SCOPE: Talent making the incision.

2.2.1 Added shot: A cutting the peritoneum

2.3. Wrap the intestine with wet gauze and move it gently to the outside of the body to reveal the retroperitoneal organs **[1]**. Insert a notch between the erector muscles of the spine and the adipose tissue with surgical scissors, then bluntly separate them with fingers **[2-2a]**. *Videographer: This step is important!*

2.3.1. Talent wrapping and removing the intestine.

2.3.2. SCOPE: Talent inserting the notch and separating the erector muscle and adipose tissue.

2.3.2a Added angle also still photos

2.4. Carefully strip off the connective tissue around the thoracic duct **[1]**. Expose the thoracic duct from just beneath the left crus of the diaphragm caudally until 20 millimeters of the duct is visible **[2]**. Gently separate adhesions between the thoracic duct and aorta using precision tweezers or wet cotton swabs **[3]**. *Videographer: This step is important!*

2.4.1. SCOPE: Talent stripping off the connective tissue around the thoracic duct. shot all three steps together. 2.4.1 a new angle also photos

2.4.2. SCOPE: Talent exposing the duct.

2.4.3. SCOPE: Talent separating the adhesions between the duct and the aorta.

2.5. Apply a ligature on the thoracic duct just beneath the left crus of the diaphragm, causing the caudal thoracic duct to become distended **[1-TXT]**. Make a 5-millimeter hole in the abdominal wall by stabbing it with the surgical scissors, pass the hairpin-curved cannulation tube into the hole, and ligate the tube on the iliopsoas muscle **[2]**. *Videographer: This step is difficult and important!*

2.5.1. SCOPE: Talent applying a ligature and duct distending. **TEXT: 3-0 silk** NOTE: 2.5.1 a new angle, 2.5.2 b closing, 21.5.1 c run thread under thoracic duct

2.5.2. SCOPE: Talent making the hole, passing the cannulation tube, and ligating it.

2.6. Fill the cannulation tube with normal saline containing 10 units per milliliter heparin **[1]**. After placing a string beneath the distended thoracic duct, stab the duct with the sharply cut edge of the cannulation tube, cannulate it approximately 5 millimeters toward the tail, and ligate the thoracic duct with the cannulation tube **[2]**.

2.6.1. Talent filling the cannulation tube with saline and heparin.

2.6.2. SCOPE: Talent cannulating and ligating the thoracic duct. NOTE: Still photos

2.7. To prevent dehydration, create a 3-millimeter hole on the anterior wall of the antrum of the stomach using precision tweezers **[1]** and pass a 2-millimeter silicon tube to the duodenum through the pyloric ring **[2]**.

2.7.1. SCOPE: Talent creating the hole in the stomach. NOTE: 2.7.1 – 2.7.2 shot together

2.7.2. SCOPE: Talent passing the silicone tube through the hole.

2.8. After closing the wound, maintain the animals in Bollman's cages and start to infuse sugar-laden saline into the rat's duodenum from the silicon tube at a flow rate of 3 milliliters per hour **[1]**. Cover the entire cage with a paper towel to keep it warm **[2]**.

2.8.1. Talent infusing the sugar-laden saline.

2.8.2. Talent covering the cage with a paper towel.

2.9. To collect the thoracic duct lymphocytes, fix the cannulation tube to the hole in the center of the lid of a 50-milliliter conical tube on ice containing 6 units per milliliter heparin, 10% fetal bovine serum, and RPMI 1640 medium **[1]**.

2.9.1. Talent fixing the cannulation tube to the conical tube.

2.10. Replace the conical tube with the lymph fluid every 12 hours and store the fluid in a refrigerator **[1]**. ~~**[2]**.~~

2.10.1. Talent replacing the collection tube.

2.10.2. ~~Talent flushing the saline.~~

3. Lymphocyte Labeling and Experimental Setup for Microvascular Studies

3.1. Dissolve CFDSE in dimethyl sulfoxide to 15.6 millimolar **[1]** and incubate 1 x 10^9 lymphocytes in 50 milliliters of RPMI 1640 with 50 microliters of CFDSE solution for 30 minutes at 37 degrees Celsius **[2]**.

3.1.1. Talent dissolving the CFDSE in the DMSO. NOTE: 3.1.1 – 3.1.2 shot together

3.1.2. Talent adding the CFDSE to the cells.

3.2. Under continuous anesthesia with 2% isoflurane, place the Wister Rat on a portable stainless-steel plate with a rectangle hole around the center covered with a glass slide, open the abdomen of the recipient Wister rat via a midline incision **[1]** Select approximately 10 centimeters of the idleal segment with Peyer's Patches for observation **[3]**. *Videographer: This step is important!*

3.2.1. Talent opening the rat's abdomen.

3.2.2. ~~Talent putting the rat on the platform.~~

3.2.3. Talent selecting the segment of the intestine for observation. NOTE: 2 takes

3.3. Keep the intestine moist and as warm as possible by soaking the gauze that is used to cover the small intestine with PBS warmed to 37 degrees Celsius **[1]**.

3.3.1. Talent soaking the gauze with PBS.

3.4. Put the slide on the stage of the microscope and choose suitable areas for observation of the microcirculation in Peyer's Patches where some high endothelial venules are running through the serosa **[1]**.

3.4.1. Talent selecting the area for observation. NOTE: shot together up to 3.5.3

3.5. Cover the adjacent intestinal segment and mesentery with absorbent cotton soaked with PBS **[1]**. Place the intestine segment between two rolled cotton balls **[2]** and position it as far as possible from the rat's body to prevent it from being vibrated by the rat's heartbeat and breathing **[3]**. *Videographer: This step is difficult and important!*

3.5.1. Talent covering the adjacent intestinal segment.

3.5.2. Talent placing the intestinal segment between 2 cotton balls.

3.5.3. Talent positioning the intestine.

3.6. Use a 1-milliliter syringe to slowly inject the CFSE-labeled thoracic duct lymphocytes into the jugular vein of the recipient rats from the venous catheter **[1]**.

3.6.1. Talent injecting the lymphocytes.

NOTE: first take too fast

4. Microcirculation of Lymphocytes

4.1. Continuously monitor thoracic duct lymphocytes in the microvasculature of the Peyer's Patches using CLSM and perform time-lapse imaging for 3 hours at 30-second intervals **[1]**.

4.1.1. Talent at the computer adjusting settings for the time-lapse imaging.

4.2. Inject 25 milligrams per kilogram Texas Red-dextran into the jugular vein of the recipient rat to stain the bloodstream and 5 milligrams per kilogram Hoechest 33342 to stain the cell nuclei **[1]**.

4.2.1. Talent injecting the dyes into the rat.

4.3. To quantify the lymphocyte dynamics, define lymphocytes adhering to the high endothelial venules more than 30 seconds as “adhesive lymphocytes” and those emigrating from the high endothelial venules to stroma as ‘‘migrating lymphocytes”. Then, calculate the average percentage of migration in each field of view **[1-TXT]**

4.3.1. Talent at the computer quantifying dynamics. **TEXT: migrating lymphocytes/adhesive lymphocytes + migrating lymphocytes** NOTE: Can use image マイクロ評価画像.tif here

Results

5. Results: Microscopic Images of Peyer's Patches from Rats

5.1. This protocol was used to collect naïve gut-tropic lymphocytes and observe their migration in rat Peyer's patches. Microscopic images of the Peyer's patches are shown here **[1]**.

5.1.1. LAB MEDIA: Figure 4 A.

5.2. The nuclei of vascular endothelial cells are stained blue **[1]**, the blood plasma is red **[2]**, and the blood flow is detected by the flow of unstained cells **[3]**. Gut-tropic lymphocytes, stained green, can be seen adhering to high endothelial venules **[4]**.

5.2.1. LAB MEDIA: Figure 4 B. *Video Editor: Emphasize the blue.*

5.2.2. LAB MEDIA: Figure 4 B. *Video Editor: Emphasize the red.*

5.2.3. LAB MEDIA: Figure 4 B.

5.2.4. LAB MEDIA: Figure 4 B. *Video Editor: Emphasize the green.*

Conclusion

6. Conclusion Interview Statements

6.1. **Kazuki Horiuchi:** When performing this protocol, keep in mind that the preparation to create the optimal environment for cannulation is important, including exposure of the thoracic duct and adjustment of the positional relationship between the thoracic duct and the catheter running parallel.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.5.1.*

6.2. **Kazuhiko Shirakabe:** Observing lymphocyte dynamics in the Peyer's patches under several conditions will be a foundation for future research of gut immunity.

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