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Title: Multiphoton Microscopic Observation of Vessels in Mouse Liver Tissue

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Author Questionnaire

1. Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

Videographer: Authors unable to capture, please film

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **46**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Ru Li**: This method can help us to visualize the lumens of blood vessels within the mouse liver, allowing evaluation of the dynamics of the liver in multiple dimensions **[1]**.

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

REQUIRED:

1.2. **Ziqing Wang**: Using this method, we can visualize the dynamics of blood vessels within the liver in 3 dimensions **[1]**.

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

OPTIONAL:

1.3. **Huang Junhao**: We have demonstrated the use vascular dyes, but you can perform the vessel staining with your substance of interest before microscopic visualization **[1]**.

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

Ethics Title Card

1.4. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at China Nanfang Hospital.

Protocol

2. Mouse Preparation

2.1. After confirming a lack of righting reflex in an anesthetized, 8-week old, male C57BL/6 (C-fifty-seven-black-six) mouse **[1-TXT]**, wipe the mouse's tail with 75% alcohol **[2]** and use a 1-microliter syringe equipped with a 30-gauge needle to inject 10 milligrams/milliliter of freshly prepared Rhodamine B isothiocyanate-dextran in 100 microliters of rhodamine B isothiocyanate-dextran into the caudal tail vein **[3]**.

2.1.1. WIDE: Talent checking righting reflex *Videographer: More Talent than mouse in shot* **TEXT: Anesthesia: sodium pentobarbital 50 mg/kg i.p.**
2.1.2. Mouse being held belly side up
2.1.3. Skin being wiped/sprayed

2.2. When all of the solution has been delivered, use a cotton swab to apply pressure to the puncture site **[1]** and use a gauzed wet with sterile water to soak the abdominal fur **[2]**.

2.2.1. Pressure being applied *Videographer: Important/difficult step*
2.2.2. Fur being wiped *Videographer: Important/difficult step*

2.3. Use a razor to shave the abdomen, making strokes in the direction of the fur **[1]** and place the mouse onto a 37-degree Celsius, alcohol-disinfected heating pad **[2]**.

2.3.1. Fur being wiped
2.3.2. Talent placing mouse onto heating pad *Videographer: More Talent than mouse in shot*

3. Liver Imaging Framing

3.1. To fix the mouse liver with a body organ imaging frame, first place a clean, 5-millimeter-diameter suction cup in a fixed position **[1-TXT]** and wipe the heating pad and suction cup with 75% alcohol **[2]**.

3.1.1. WIDE: Talent placing cup into position **TEXT: As of Spring2020, commercial organ imaging frame has not been released**
3.1.2. Talent wiping pad and/or cup, with alcohol container visible in frame

3.2. Connect the suction cup to the vacuum pump hose **[1]** and turn on the pump **[2]**.

3.2.1. Talent connecting cup to hose
3.2.2. Talent turning on pump

3.3. On a 75% alcohol-sterilized table, use surgical scissors to cut 2 centimeters of skin from the lower sternal border of the mouse **[1]** and expose the liver **[2]**.

3.3.1. Skin being cut *Videographer: Important step*
3.3.2. Liver being exposed *Videographer: Important step*

3.4. Place the mouse and the heating pad onto the holder base of the body frame **[1]** and adjust the organ imaging fixture so that the suction cup can hold the liver **[2]**.

3.4.1. Mouse being placed into frame *Videographer: Important/difficult step*
3.4.2. Fixture being adjusted *Videographer: Important/difficult step*

3.5. Then use a negative pressure of 30-35 kilopascals for suction so that the liver attaches to the cup **[1]**.

3.5.1. Liver being suctioned to cup *Videographer: Important step*

4. Multiphoton Laser Scanning Microscope Setup

4.1. To set up the multiphoton laser scanning microscope, turn on the microscope **[1]** and select the 60X objective **[2]**.

4.1.1. WIDE: Talent turning on microscope
4.1.2. Talent selecting objective

4.2. Fix the frame and mouse under the objective **[1]** and add a drop of normal saline to the cup large enough to cover the lens **[2]**.

4.2.1. Talent fixing frame/mouse *Videographer: More Talent than mouse in shot*
4.2.2. Drop being added to lens

4.3. Adjust the objective so the lens just touches the normal saline **[1]** and turn on the laser software **[2]**.

4.3.1. Lens being moved to touch saline
4.3.2. Talent turning on software, with monitor visible in frame

4.4. Turn on the laser **[1]** and press and hold the power button and shutter for 3 seconds **[2]**.

4.4.1. Talent turning on laser
4.4.2. Talent pressing/holding power button and/or shutter

4.5. The start the microscope operating software **[1]** and set the laser to 800 nanometers **[2]**.

4.5.1. Talent starting microscope software, with monitor visible in frame
4.5.2. SCREEN: Laser being set to 800 nm

4.6. To set the **Image Acquisition Control**, click the **Fluorescent** switch **[1]** and turn off the room and equipment lights **[2]**.

4.6.1. Talent clicking switch
4.6.2. Talent turning off lights

4.7. Open the light path shutter on the microscope **[1]** and rotate the fluorescence filter to the fourth gear **[2]**.

4.7.1. Talent opening shutter
4.7.2. Talent rotating filter

4.8. Pull the two levers of the optical switch **[1]** and, looking through the eyepiece, use the coarse and fine focusing quasispirals to adjust the focal length **[2]**.

4.8.1. Talent pulling lever(s)
4.8.2. SCREEN: Focal length being adjusted

4.9. Then use the **XY** axes to adjust the field of view to locate the target area **[1]**.

4.9.1. SCREEN: Field of view being located

5. Multiphoton Laser Scanning Micrography

5.1. For imaging, turn off the fluorescent switch in the software **[1]** and switch the fluorescent filter to the second gear **[2]**.

5.1.1. WIDE: Talent turning off switch, with monitor visible in frame
5.1.2. Talent switching to second gear

5.2. Push the two levers of the optical switch **[1]** and click **Focus ×2** to preview the target area **[2]**.

5.2.1. Talent pushing levers
5.2.2. SCREEN: Focus x 2 being clicked/target being previewed

5.3. Adjust the acquisition settings and the image acquisition control parameters **[1]** and press **Control + C** to adjust the high voltage, gain, and offset **[2]**.

5.3.1. SCREEN: Settings being adjusted
5.3.2. SCREEN: Control + C being pressed/voltage, gain, and offset being adjusted

5.4. Click **Stop** to stop the preview, click the **XY** button to scan in two dimensions, and click **Save [1]**.

5.4.1. SCREEN: Stop and CY being clicked, then image being saved

5.5. Select a region and click **Depth** and **Preview** to select the end and start sets in the **Microscope** settings **[1]**.

5.5.1. SCREEN: Region being selected, then Depth and Preview being clicked

5.6. Scan 3D images **[1]** and save **[2]**.

5.6.1. SCREEN: Image(s) being scanned
5.6.2. SCREEN: Image(s) being saved

5.7. After scanning, select a region, click **Time**, and adjust the other acquisition settings and image acquisition control parameters **[1]**.

5.7.1. SCREEN: Region being selected, Time being clicked, parameters being set

5.8. Then scan through the different slices **[1]** and save to obtain the resulting image movie **[2]**.

5.8.1. SCREEN: Slice(s) being scanned
5.8.2. SCREEN: Movie being saved

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.
2.2., 3.3.-3.5.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.
2.2. The tail vein is very small, so watch carefully as you inject.
3.4. Do not damage organs or cause excessive bleeding when cutting the skin.

Results

6. Results: Representative Body Organ Frame Imaging

6.1. Here visualization of the distribution of blood vessels within the liver using multiphoton microscopy as demonstrated can be observed **[1]**.

6.1.1. LAB MEDIA: Figure 1

6.2. This blood vessel is divided into a plurality of branches **[1]** emanating from a trunk **[2]** and distributed into the surrounding space **[3]**.

6.2.1. LAB MEDIA: Figure 1 *Video Editor: please emphasize branches*
6.2.2. LAB MEDIA: Figure 1 *Video Editor: please emphasize trunk*
6.2.3. LAB MEDIA: Figure 1

6.3. The outer circumference of the blood vessel can be observed in red **[1]**, while the inner cavity is dark **[2]**.

6.3.1. LAB MEDIA: Figure 1 *Video Editor: please emphasize outer circumference in at least one part of vessel*
6.3.2. LAB MEDIA: Figure 1 *Video Editor: please emphasize inner cavity in at least one part of vessel*

6.4. As observed in the video, non-red objects moving within the vessels could be cells **[1]**.

6.4.1. LAB MEDIA: Video 1

6.5. The darkness of the tissue observed in this video is likely due to the liver not having been fixed well over time **[1]**.

6.5.1. LAB MEDIA: Video 1 00:02 *Video Editor: please emphasize darkened areas in right of image*

Conclusion

7. Conclusion Interview Statements

7.1. **Ru Li**: It is important to remember that how securely the liver is fixed affects the stability of the image **[1]**.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (3.5.)

7.2. **Ru Li**: This methodology can also be applied to the positioning of other structures within the liver for the direct observation of the distribution or changes in specific liver structures of interest **[1]**.

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