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Multiphoton microscopic observation of vessels in mouse liver tissue

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TITLE

Multiphoton Microscopic Observation of Vessels in Mouse Liver Tissue

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KEYWORDS:

Mouse, Liver, Multiphoton microscope, Blood vessel, Living observation, Organ imaging fixture

SUMMARY:

In this experiment, a mouse is injected in its tail vein with Rhodamine B isothiocyanate–dextran that can stain blood vessels. After the liver is exposed and fixed, a specific part of the liver can be selected to observe the deep tissue in the living body using multiphoton microscopy.

ABSTRACT:

Observing the intravascular dynamics of mouse liver tissue allows us to conduct further in-depth observations and studies on tissue-related diseases of the mouse liver. A mouse is injected with a dye that can stain blood vessels. To observe the mouse liver in vivo, it is exposed and fixed in a

frame. Two and three-dimensional images of the blood vessels in the liver tissue are obtained using a multiphoton microscope. Images of the tissues at the selected sites are continuously acquired to observe long-term changes; the dynamic changes of blood vessels in the liver tissues are also observed. Multiphoton microscopy is a method for observing cell and cell function in deep tissue sections or organs. Multiphoton microscopy has sensitivity to tissue microstructure and enables imaging of biological tissues at high spatial resolution in vivo, providing the ability to capture the biochemical information of the organization. Multiphoton microscopy is used to observe part of the liver but fixing the liver to make the image more stable is problematic. In this experiment, a special vacuum suction cup is used to fix the liver and obtain a more stable image of the liver under the microscope. In addition, this method can be used to observe dynamic changes of specific substances in the liver by marking such substances with dyes.

INTRODUCTION

Blood vessels can provide nutrients for various organ tissues of the human body, and exchange substances. At the same time, many cytokines, hormones, drugs, and cells also function through vascular transport to specific locations. Observing vascular changes in liver tissue can help in understanding the distribution of blood flow in liver tissue and the transport of substances, and assist in the analysis of certain vascular-related diseases[1,2].

There are many ways to observe the blood vessels of the liver in mice. Among them, optical microscopy has many limitations in observing opaque vascular tissue[3]. Multiphoton microscopy can be used to image the blood vessels of living livers with noninvasive high resolution[4]. Not only can three-dimensional images of blood vessels be obtained, but the technique can also be used to help organize the tissue to observe biological effects therein; furthermore, the whole tissue can be imaged rather than only the microvessels as in computed tomography and magnetic resonance imaging[5].

Multiphoton microscopy can be used to effectively detect scattered fluorescent signals in deep living tissue, with less phototoxicity[6]. Therefore, the activity of living tissue can be ensured, and the amount of damage can be reduced. Multiphoton microscopy has better penetrating power than confocal microscopy, allowing deeper layers to be observed[7], providing unique 3D imaging. Multiphoton microscopy is now often used in imaging cranial nerves[8] and has been extended to the study of neuronal dynamics in live mice[9-11].

In this experiment, after fluorescent labeling of mouse blood vessels, the liver is fixed in a frame, and the dynamics of blood vessels in living liver tissue can be seen using multiphoton microscopy. This experiment demonstrates how to mark specific substances, use multiphoton microscopy to help observe a location within the tissue, observe cellular events in the intercellular tissue, make photochemical measurements[12-14], and observe the material dynamics inside the living tissue[15].

For example, tumor endothelial marker 1 (TEM1) has been identified as a novel surface marker upregulated on the blood vessels and stroma in many solid tumors, marking single-chain variable fragment (scFv) 78 against TEM1, and then multiphoton microscopy can be used for mouse hemangioma location and evaluation of tumors[16].

PROTOCOL:

All animal care and procedures were in accordance with China Nanfang Hospital policies for heath and well-being (application No: NFYY-2019-73).

1. Mouse preparation

1.1. Anesthetize the mouse.

1.1.1. Prepare sodium pentobarbital (50 mg/kg) in a syringe.

1.1.2. Grab the mouse (8-week-old male C57BL/6) with the left hand so that its belly is facing up and its head is lower than its tail. Disinfect the abdominal skin with 75% alcohol.

1.1.3. Holding the syringe in the right hand, pierce the abdomen white line with the needle slightly to the right side of the skin by 3-5 mm. Ensure that the syringe needle and the skin are at a 45° angle, inject the pentobarbital slowly into the abdominal cavity.

1.1.4. Check whether the anesthesia was successful by the lack of a righting reflex.

1.2. Inject Rhodamine B isothiocyanate–dextran into the caudal vein.

1.2.1. Prepare 100 µL of 10 mg/mL Rhodamine B isothiocyanate–dextran in a syringe.

1.2.2. Wipe the mouse's tail with 75% alcohol.

1.2.3. Hold the tail with the left hand, hold the syringe in the right hand with the needle parallel to the vein, and inject the 100 µL dye.

1.2.4. Stop the bleeding with a cotton swab after the injection.

1.3. Soak the mouse's abdominal fur with gauze wetted with sterile water, and shave the mouse's abdomen using a razor, making strokes in the direction of the fur.

1.4. After wiping a heating pad with 75% alcohol, open the heating pad, and place the mouse on its back in the heating pad to maintain its body temperature at 37 °C.

2. Fixing the mouse liver with the body organ imaging frame

NOTE: The commercial organ imaging frame has not been released yet.

2.1. Place a clean suction cup in a fixed position.

2.2. Install an organ imaging fixture (**Supplementary Figure 1**) and wipe the heating pad and suction cup with 75% alcohol.

2.3. Connect the suction cup (5 mm diameter) to the vacuum pump hose and turn on the vacuum pump.

2.4. On a sterile table sterilized with 75% alcohol, use surgical scissors to cut 2 cm of skin off the lower sternal border of the mouse and expose the liver.

2.5. Place the mouse together with the heating pad on the holder base.

2.6. Adjust the organ imaging fixture so that the suction cup holds the liver. Then use a negative pressure of 30-35 kPa for suction so that the liver attaches to the suction cup (**Supplementary Figure 2**).

3. Adjusting the multiphoton laser scanning microscope

3.1. Turn on the multiphoton microscope and select the 60x/1.00 W objective.

3.2. Fix the frame and mouse under the objective lens.

3.3. Add a drop of normal saline that can cover the entire lens, which is the center of the suction cup, and adjust the objective lens to just touch the normal saline.

3.4. Double-click on the computer icon to turn on the laser software and turn on the switch. Then press and hold the power button and shutter for 3 s.

3.5. Set the wavelength to 800 nm.

3.6. Start the microscope operating software using the computer.

3.7. For the **Acquisition Setting**, set **Laser 800**.

4. Observation using multiphoton laser scanning microscope

4.1. For **Image Acquisition Control**, click the **Fluorescent** switch.

4.2. Ensure that the room and equipment lights are switched off. To reduce noise levels, use the microscope in a darkened environment.

4.3. Open the light path shutter on the microscope.

4.4. Rotate the fluorescence filter to the fourth gear and pull the two levers of the optical switch.

4.5. Looking through the eyepiece, use the coarse and fine focusing quasispirals to adjust the focal length, and use the *X*/*Y* axes to adjust the field of view to find the target area.

5. Multiphoton laser scanning micrography

5.1. Turn off the fluorescent switch on the software, switch the fluorescent filter to the second gear (second gear is RXD1), and push the two levers of the optical switch.

5.2. Click on **Focus ×2** to preview the target area and adjust the acquisition setting and image acquisition control parameters (image resolution: 1024).

5.3. Press **Control + C** and adjust the high voltage, gain, and offset (to reduce noise levels).

5.4. Click **Stop** to stop preview, click the **XY** button to scan in two dimensions, and save after completion (**Figure 1**).

5.5. Select a region, click on **Depth**, and then **Preview** to select the end set and start set (see the complete required part of the blood vessel) in the **Microscope** setting. Scan 3D images and save after completion (**Figure 2**).

5.6. Select a region, click on **Time**, and adjust other acquisition setting and image acquisition control parameters (Step Size: 1 μm; Slices: 10; Time Scan Number: 40). Scan different slices and save after completion to obtain moving images (**Video 1**).

NOTE: Have someone care for the mice during the scan and add anesthesia accordingly.

5.7. Sacrifice the mouse by neck dissection.

REPRESENTATIVE RESULTS

The distribution of blood vessels in the liver can be seen in **Figure 1**, obtained using multiphoton microscopy. The blood vessel is divided into a plurality of branches emanating from a trunk and distributed to the surrounding space. The outer circumference of the blood vessel is red, the inner cavity is dark, and there are many things inside. The clearer the image, the closer to the plane of observation it is. There are also some red spots around, probably because the dye penetrates the surrounding tissue to stain other substances. In **Video 1**, there are non-red things that could be cells moving in the red blood vessels. At the end of the video, some of these things are darkened. This is probably because the fixed liver has not been fixed well over time. This method will allow imaging for 2 hours.

FIGURE AND TABLE LEGENDS:

Figure 1: The blood vessels in the liver tissue under multiphoton microscopy. The blood vessels are red. A two-dimensional image of the blood vessel with a resolution of 2048 and an HV of 512, can clearly image the red blood vessels after staining. There are also some red spots around, and the dye may also penetrate the surrounding tissue to dye other substances.

Figure 2: Three-dimensional image of living liver tissue in the same position. The thickness is 9 μm, the morphology of the blood vessels is observed from different angles, and dynamic images are collected. After synthesis, the cells in the blood vessels (dark) are seen flowing.

Supplementary Figure 1: Body organ imaging frame. (**a**) Heating pad: It maintains the mouse body temperature. (**b**) Bracket: It can provide a base to place the mouse and the adjustable suction cup position with knobs. (**c**) Suction cup: It is fixed by the bracket. The negative pressure provided by the negative pressure pump causes the liver to adsorb to the transparent lens in the center of the suction cup. (**d**) Flexible pipe: It connects the suction cup with the pressure pump. The pressure pump provides negative pressure to the suction cup. (**e**) Centrifuge tube: The centrifuge tube in the middle of the flexible pipe can hold the liquid, to prevent these liquids from being sucked into the vacuum pump. (**f**) Negative pressure pump: It is connected to the suction cup by the flexible pipe. The negative pressure pump can provide negative pressure to the suction cup to make the liver cling to the lens.

Supplementary Figure 2: A close-up image of the liver attached to transparent lens. The transparent lens is in the center of the suction cup. The pump provides negative pressure to the suction cup to make it suck the liver and the liver is attached to the transparent lens. Because

the suction-faced liver is a bit away from the mouse, the effect of breathing and the heartbeat on artifacts is reduced. The transparent lens can provide a stable view for the multiphoton microscope.

DISCUSSION:

Observing a specific living tissue is an effective means of understanding the changes, localization, and biological effects of the material inside the tissue[17]. In this experiment, the important steps are fixing the liver with an organ imaging fixture, which can solve the problem of motion artifacts due to breathing and heartbeats, and the use of a multiphoton microscope for observation. Using this method, the internal tissues of the liver in vivo are observed through a multiphoton microscope, and the blood vessels are fluorescently labeled to clearly observe their position and three-dimensional structure. This method can be used to observe specific effects by fluorescently labeling a substance and dynamically following the substance in the living tissue to provide further visualizations.

Multiphoton microscopy has evolved from being a novel technique to become an indispensable tool for collecting information from cellular events in an organized tissue environment. However, the organ must be fixed in place to produce a stable image so that the method can be effective in observing certain tissues and organs. Movement artifacts from breathing and the heartbeat can prevent the liver from being directly imaged, so a body organ fixture is used, which uses the principle of vacuum adsorption to secure the organ and prevent image shake. The vacuum chuck can also fix a variety of body organs. However, there is a risk of tissue rupture if the appropriate negative pressure is not used or the imaging time extends beyond 2 h.

Although the resolution of multiphoton microscopy is slightly less than that of confocal microscopy, the depth of penetration is increased to 9-10 μm, enabling the provision of images for deeper tissue observation, cellular events, and material effects.

ACKNOWLEDGMENTS

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DISCLOSURES

The authors have nothing to disclose.

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Figure

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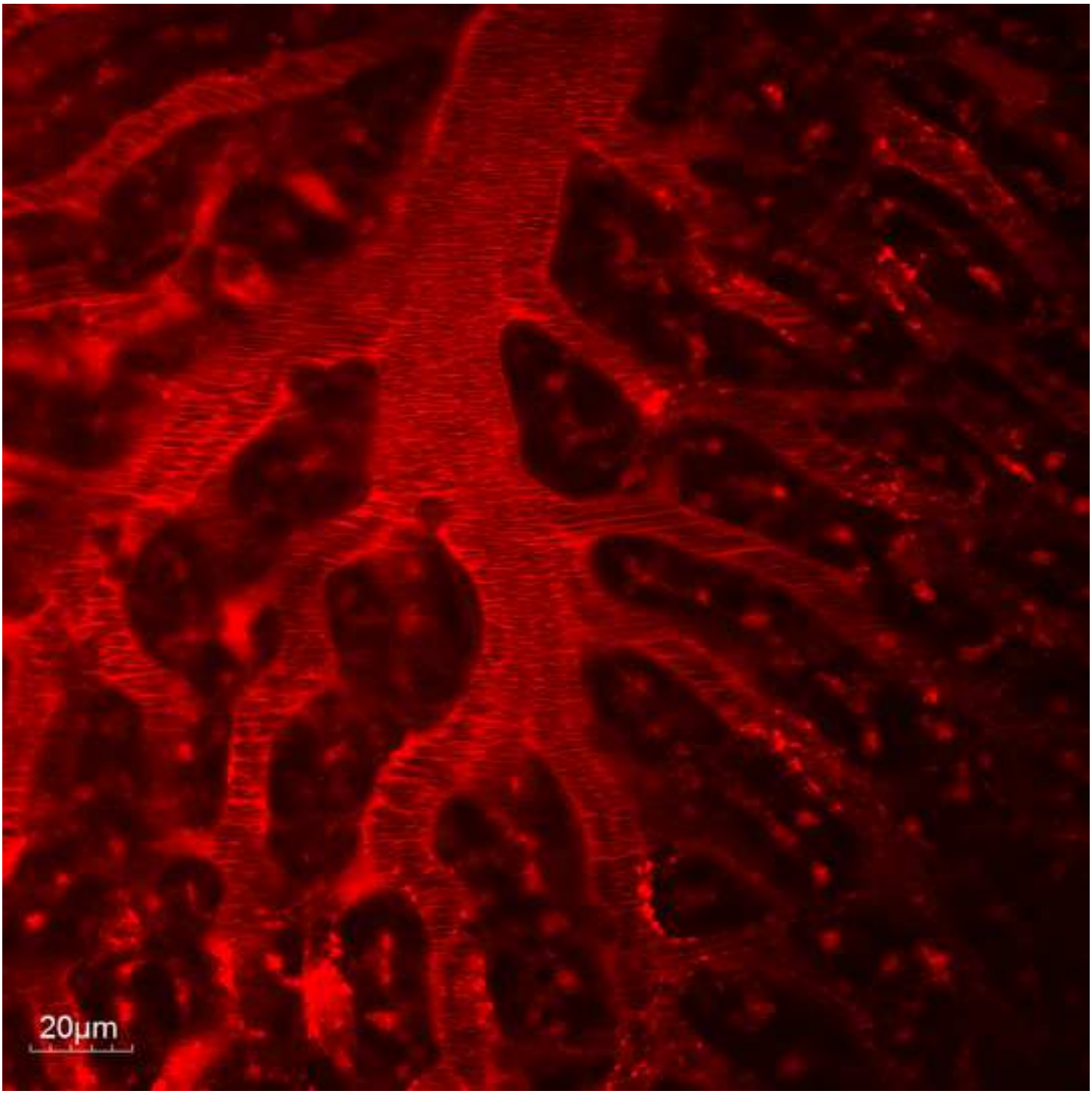

Figure

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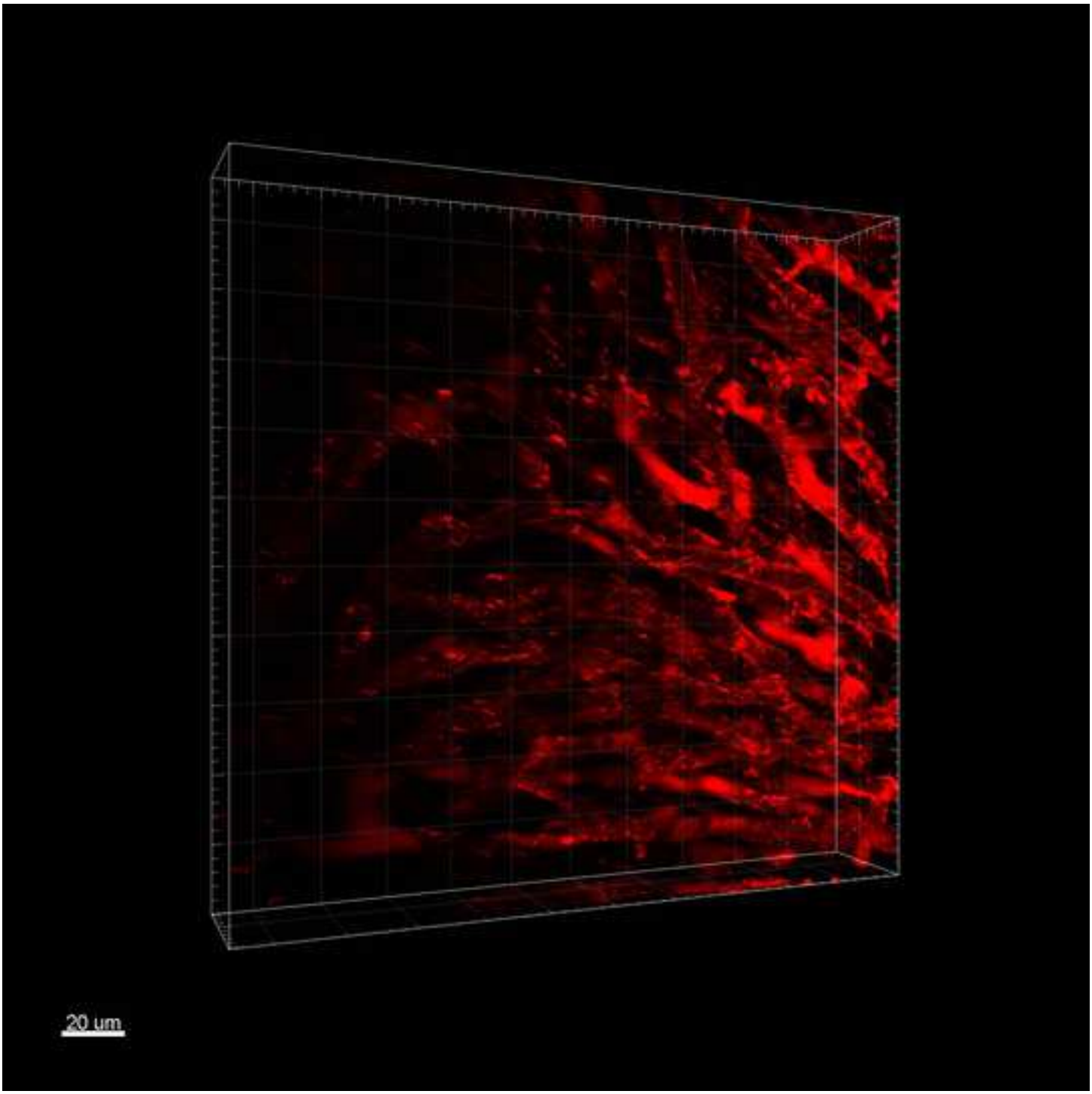
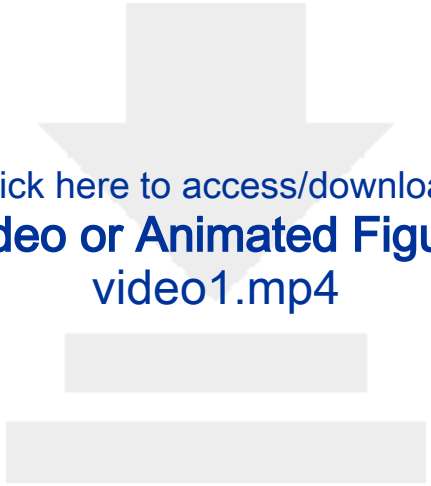


video



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Video or Animated Figure

video1.mp4

Table of Materials

Name of Material/Equipment	Company	Catalog Number	Comments/Description
1 mL syringe x 2	Hunan Pinan Medical Devices Technology	YA0551	
5 W heating pad	BiolinkOptics Techn	BL336	
75% absolute ethanol	Guangdong Guanghua Sci-Tech	1.17113.023	
Absorbent cotton ball	Healthy Sanitation Kingdom		
Mouse surgical instrument	RWD Life Science	SP0001-G	Including scissors and tweezers
Multiphoton microscopy	Olympus	FV1200MPE	
Organ imaging fixture	BiolinkOptics Techn	BL336	Including suction cup, hose, negative pressure pump and b
Rhodamine B isothiocyanate–Dextran	Sigma	R9379	
Shaving machine	Lei Wa	RE-3201	
Sodium pentobarbital	Sigma	P3761-25G	

›racket

Dear editor,

Thanks for your comments. My manuscript, JoVE60932 "Multiphoton microscopic observation of vessels in mouse liver tissue," has been modified according to the comments.

I have added two figures to explain the organ imaging frame, and both of them are 300 dpi. I have retained the original resolution of the other two pictures observed under the multiphoton microscope.

Best regards,

Rongrong Wen

Dear Reviewer 1:

Thank you very much for your valuable comments. I have submit two figures in order to clearly explain the organ imaging frame. One is all parts of organ imaging frame. The other is a close-up image of the liver attached to the suction cup. The company which

product organ imaging fixture told us this organ imaging fixture has not been officially launched yet, so there is less information about it online.

Thanks again for your suggestions

Best regards,

Rongrong Wen

Dear Reviewer 2:

Thank you very much for your comments. I added a picture to show components of the organ imaging fixture and the close-up image for the liver. The transparent lens in the center of the suction cup is equivalent to a glass slide, dripped physiological saline on it, and then adjusted the microscope head to form an immersion lens to observe. An explanation has been made about what happened later in the video, because the time is too long and the liver cannot be sucked up by the vacuum suction cup very well, and it

falls off a bit. If there will be risk of tissue rupture depend on the strength of the negative pressure pump, and the general imaging time is preferably 2 hours. Here is the use of organ fixation to control the artifacts caused by breathing and heartbeat, because negative pressure can stably absorb the liver on the lens of the suction cup and there is a certain distance between the suction cup and the mouse body. 9 ~ 10μm is the depth range of this area. For this range, we can only see the whole of some microvessels. If we want to see some thick blood vessels, it is really a little shallow.

Thanks again for your suggestions

Best regards,

Rongrong Wen

Supplementary Figure 1

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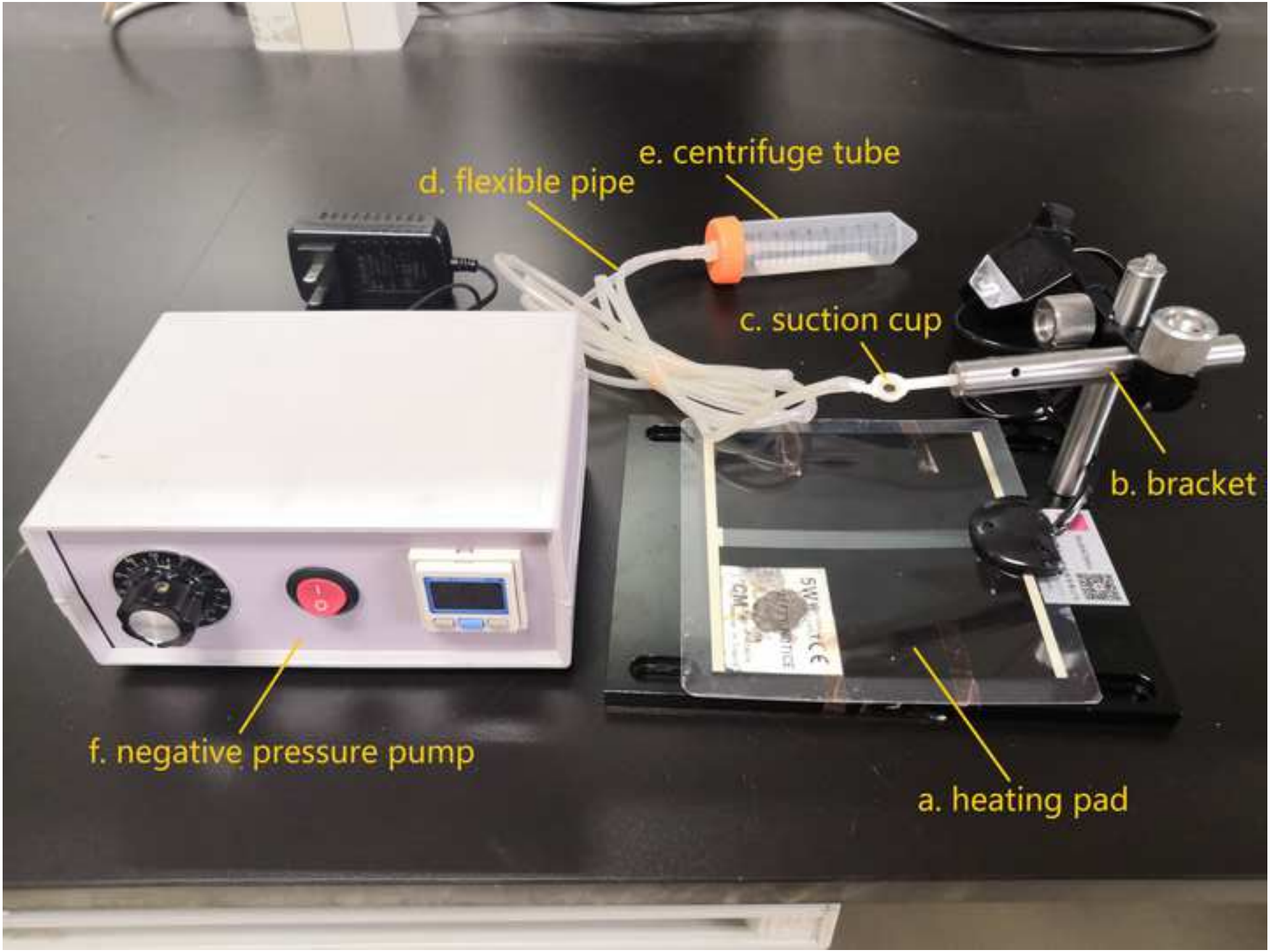

Supplementary Figure 2

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