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Title: Ultrasound Localization Microscopy for Super-Resolution Mapping of the Rodent Brain Microvasculature

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Author Questionnaire

- 1. Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**
- 2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**
- 3. Filming location:** Will the filming need to take place in multiple locations? **No**
- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 31

Number of Shots: 55

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

~~What is the scope of your research? What questions are you trying to answer?~~

- 1.1. **Sara Romanzi**: Our research aims to characterize the brain's microvascular architecture and flow, while refining super-resolution ultrasound methods.
 - 1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~What are the most recent developments in your field of research?~~

- 1.2. **Sara Romanzi**: Recent developments in our research field have optimized microbubble tracking and strengthened processing pipelines for high-resolution vascular mapping with ULM.
 - 1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What significant findings have you established in your field?~~

- 1.3. **Pia Pelaez**: This work establishes that ultrasound localization microscopy can reliably produce super-resolution maps of microvascular structure and flow *in vivo*.
 - 1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~What research gap are you addressing with your protocol?~~

- 1.4. **Pia Pelaez**: This protocol fills the need for a standardized procedure that enables reliable ULM acquisitions in living rodents using a dedicated fUS platform.
 - 1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How will your findings advance research in your field?~~

- 1.5. Pia Pelaez: These findings establish a dependable protocol that allows researchers to capture microvascular dynamics with high precision and consistency.

Videographer's Note: Delete 1.5

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been approved the local ethics committee (Comité d'éthique en matière d'expérimentation animale number 59, 'Paris Centre et Sud', project #2020-16)

Protocol

2. Animal Preparation and Tail Vein Catheterization

Demonstrator:

NOTE: Demonstrators names have not been provided

- 2.1. To begin, place an anesthetized mouse onto a stereotaxic frame with a heating pad set to 37 degrees Celsius [1-TXT].
 - 2.1.1. WIDE: Talent placing the anesthetized mouse into the stereotaxic frame over the heating pad. **TXT: Anesthesia: 2% isoflurane with air and O₂**
Videographer's Note: Shot in MED +CU
- 2.2. Using firm but non-damaging pressure, pinch the toe or tail to check for the absence of reflexes and confirm adequate anesthesia depth [1-TXT]. Apply eye ointment to both eyes to prevent cataract formation during the procedure [2].
 - 2.2.1. Talent gently pinch the tail of the mouse. **TXT: If reflex observed, slightly increase isoflurane and reassess**
 - 2.2.2. Talent gently applying eye ointment to the mouse's eyes using a sterile applicator.
- 2.3. Now, shave the head of the mouse using a trimmer [1]. Apply depilatory cream, let it sit for 1 to 1.5 minutes [2], then rinse with warm water and dry with gauze [2].
 - 2.3.1. Talent trimming the fur on the mouse's head using an electric trimmer.
 - 2.3.2. Talent applying depilatory cream to the shaved area.
 - 2.3.3. Talent rinsing and drying the skin with gauze. **TXT: Repeat as needed until all hair is removed; Avoid leaving cream on too long**
- 2.4. Next, apply centrifuged ultrasound gel onto the head to ensure acoustic coupling [1].
 - 2.4.1. Talent applying a layer of ultrasound gel evenly across the mouse's scalp.
- 2.5. For tail vein catheterization, use a 27-gauge by half-inch winged infusion set connected to an 18-gauge by half-inch needle with 8 inches of tubing [1-TXT].
 - 2.5.1. Talent holding a winged infusion set and attaching it to an 18-gauge needle using 8-inch tubing. **TXT: Shorten the tubing, if possible, to minimize dead volume**

- 2.6. Gently turn the animal's tail 90 degrees to expose the lateral tail veins [1]. Then secure the tail in place with tape to maintain stability and prevent movement during catheterization [2].
- 2.6.1. Talent rotating the mouse's tail sideways to a 90-degree position.
- 2.6.2. Talent taping the tail to the work surface to hold it in place securely.
- 2.7. Now, identify the tail vein. [1]. To improve vein visibility, warm the tail with lukewarm water to promote vasodilation [2].
- 2.7.1. Shot of the tail vein.
- 2.7.2. Talent applying a warm, damp cloth to the tail to facilitate vasodilation.
Videographer's Note: Shot with 2.7.1
- 2.8. Using a finger, apply gentle pressure at the base of the tail and slide it toward the injection site against the direction of blood flow [1]. Insert the needle parallel to the vein with the bevel facing up [2].
- 2.8.1. Talent pressing at the tail base and sliding a finger toward the mid-tail region.
- 2.8.2. Shot of the needle being inserted at a shallow angle into the distended lateral tail vein with the bevel clearly positioned upward.
- 2.9. Observe the tubing for blood flashback to confirm correct needle placement [1].
- 2.9.1. Shot of blood visibly flowing back into the tubing, confirming successful vein entry. **Author's Note: Shot 2.8.2 filmed with 2.9.1**
- 2.10. On the imaging system, launch the functional ultrasound acquisition software and create a new experiment session [1]. Adjust the position of the ultrasound probe [2].
- 2.10.1. SCREEN: 8612_screenshot_1.mp4 00:00-00:26.
- 2.10.2. SCREEN: 68612_screenshot_1b.mp4 00:03-00:19.
- 2.11. Now, start the **Live View** acquisition in the software and adjust the probe in real time using power doppler imaging to center the brain on the screen [1]. Optimize the imaging parameters, such as contrast and compression, to enhance visualization quality [2].
- 2.11.1. SCREEN: 68612_screenshot_2 00:01-00:05
AND
68612_screenshot_2b 00:12-00:24; 00:34-00:47
- 2.11.2. SCREEN: 68612_screenshot_2b 00:56-01:12

2.12. Then, open the **Angio3D** (*Angio-3-D*) menu in the acquisition software [1]. Adjust the scanning parameters to fully cover the brain, then start the scan acquisition [2].

2.12.1. SCREEN: 68612_screenshot_3 00:00-00:03

2.12.2. SCREEN: 68612_screenshot_3 00:04-00:21

2.13. Keep the acquisition software open and launch the **IcoStudio** (*Ico-Studio*) software for analysis and visualization. Load the **Angio3D** scan for review [1].

2.13.1. SCREEN: 68612_screenshot_4 00:00-00:24

2.14. Navigate to the **Brain Registration** panel and register the Angio3D scan to the ~~Allen Mouse Common Coordinate Framework~~ using either the fully automatic or manual mode [1]. Save the completed registration as a **.bps** (*B-P-S*) file [1].

2.14.1. SCREEN: 68612_screenshot_5 00:00-00:08

2.14.2. SCREEN: 68612_screenshot_5 00:20-00:30

2.15. Now, open the **Brain Navigation** menu and scroll down to the **Atlas Manager** panel [1]. Use the Parent and Child tree navigator to browse the Allen Mouse Brain Atlas and select the desired anatomical regions for overlay in the 3-view panel [2].

2.15.1. SCREEN: 68612_screenshot_6 00:00-00:05

2.15.2. SCREEN: 68612_screenshot_6 00:06-00:24

2.16. Choose an imaging plane that overlaps with the selected regions of interest [1]. In the coronal view, manually place two markers to define the desired imaging slice [2].

2.16.1. SCREEN: 68612_screenshot_7 00:00-00:09

2.16.2. SCREEN: 68612_screenshot_7 00:10-00:22

2.17. Then, click on **BPS Positioning** to extract the motor coordinates for the selected imaging plane [1]. Review the image preview from the Angio3D scan and click **Copy BPS Coordinates** [2].

2.17.1. SCREEN: 68612_screenshot_8 00:00-00:04

2.17.2. SCREEN: 68612_screenshot_8 00:04-00:08

2.18. Switch to the IcoScan software and open the **Move Probe** panel [1]. Click on **Neuronavigation**, paste the copied values, and allow the system to automatically align the probe with the selected imaging plane [2].

2.18.1. SCREEN: 68612_screenshot_9 00:01-00:04

2.18.2. SCREEN:68612_screenshot_9 00:05-00:15

2.19. Then, start Live Imaging to confirm that the current imaging plane aligns with the previously selected plane [1].

2.19.1. SCREEN: 68612_screenshot_10 00:21-00:36

3. Ultrasound Localization Microscopy (ULM) Acquisition of the Brain Microvasculature

Demonstrator:

NOTE: Demonstrators names have not been provided

3.1. Using a sterile syringe, inject 5 milliliters of 0.9 percent sodium chloride solution into the vial through the septum [1]. Agitate the vial vigorously for 20 seconds to resuspend the microbubbles and ensure uniform dispersion [2].

3.1.1. Talent inserting a sterile syringe through the septum of a vial and slowly injecting 5 milliliters of sodium chloride solution.

3.1.2. Talent holding the vial and shaking it energetically for 20 seconds.

3.2. Predefine the recording parameters, including ultrasound sequence, frame rate, and total recording duration [1].

3.2.1. SCREEN: 68612_screenshot_11 00:00-00:23

3.3. Then, draw the desired volume of microbubbles for injection and begin the acquisition [1]. Once acquisition starts, inject 100 microliters for mice, using the appropriate dilution [2]. Confirm contrast enhancement on the live visualization panel [3]. Immediately flush the remaining microbubbles in the dead volume by injecting physiological fluid into the catheter [4].

3.3.1. Talent drawing microbubble solution into a syringe according to the species-specific volume. **Videographer's Note: 2sd part**

3.3.2. Talent injecting the microbubbles into the catheter.

3.3.3. SCREEN: 68612_screenshot_12 00:31-00:44

3.3.4. Talent injecting physiological fluid into the catheter to flush remaining microbubbles. **Videographer's Note: Shot with 3.3.2 stop before mouse's limb moves**

3.4. Wait for the acquisition to finish and save the recorded data [1-TXT].

3.4.1. SCREEN: 68612_screenshot_13 00:14-00:20; 00:45-00:55. TXT: **Stop isoflurane and monitor the animal in a warm area until fully awake**

4. Image Reconstruction using the IcoLab Software

Demonstrator:

4.1. Open the IcoLab software and click on the **Ultrasound Localization Microscopy** tab located on the leftmost part of the screen to access the ultrasound localization microscopy interface [1]. Then, click on **Compute ULM Maps** to begin the processing workflow [2].

4.1.1. SCREEN: 68612_screenshot_14 00:00-00:03

4.1.2. SCREEN: 68612_screenshot_14 00:03-00:05

4.2. Select the source folder that contains the previously acquired data [1]. Then, select the specific scan file to process and adjust the processing time boundaries as needed [2].

4.2.1. SCREEN: 68612_screenshot_15 00:00-00:08

4.2.2. SCREEN: 68612_screenshot_15 00:09-00:28

4.3. Click on **Next** to move to the Reporting step. Set the output folder path for saving the processed files and configure the visualization parameters for the report images [2].

4.3.1. SCREEN: 68612_screenshot_16 00:01-00:21

4.4. Now click on **RUN** to begin processing the data [1]. Once completed, a **.trk (T-R-K)** file will be created containing localized microbubble coordinates, along with TIFF files representing density, velocity, and backscattered amplitude [2]. Load the **.trk** file into IcoStudio to begin exploring the processed data [3].

4.4.1. SCREEN: 68612_screenshot_17a 00:01-00:04

4.4.2. SCREEN: 68612_screenshot_17b 00:11-00:25

4.4.3. SCREEN: 68612_screenshot_17b 00:26-00:36

4.5. Then, use the right-side panel in IcoStudio to adjust visualization parameters such as contrast, compression, and colormap for enhanced data representation [1].

4.5.1. SCREEN: 68612_screenshot_18 00:00-00:10

- 4.6. To set the integration time, adjust the double slider located at the bottom of the main view to define the start and end frames for the analysis [1]. The **Density** tab shows the distribution of microbubble occurrences. Choose between **Absolute Density** and **Directional Density** [2].

4.6.1. SCREEN: 68612_screenshot_19 00:00-00:13

4.6.2. SCREEN: 68612_screenshot_19 00:14-00:24

- 4.7. After that, click on the **Velocity** tab to display microbubble speed in millimeters per second using Absolute, Directional, Axial, and Lateral modes [1]. Adjust the contrast, compression, and velocity thresholds to improve image clarity [2].

4.7.1. SCREEN: 68612_screenshot_20 00:00-00:13

4.7.2. SCREEN: 68612_screenshot_20 00:14-00:26

- 4.8. Then explore the **BSA** tab to view microbubble reflectivity, which enhances contrast and reveals microvascular structures and out-of-plane motion [1]. Export images as high-resolution PNGs (*P-N-Geez*) or generate TIFF files for quantitative analysis with customizable pixel size [2].

4.8.1. SCREEN: 68612_screenshot_21 00:00-00:09

4.8.2. SCREEN: 68612_screenshot_21 00:18-00:34, 00:42-00:43

Results

5. Results

- 5.1. Microbubble density maps of a coronal rat brain slice acquired with ULM resolved both large vessels and fine capillary structures, revealing detailed vascular topology including bifurcations and branching patterns [1].
 - 5.1.1. LAB MEDIA: Figure 5. *Video editor: Highlight the leftmost full brain panel under "Density," then zoom in on the bottom left inset*
- 5.2. The velocity map of the same brain slice showed clear axial directional flow, with upward flow representing venous drainage and downward flow corresponding to arterial perfusion, consistent with known cortical vascular architecture [1].
 - 5.2.1. LAB MEDIA: Figure 5. *Video editor: Highlight the center full brain panel under "Velocities," then zoom in on the bottom center inset showing vessels with both blue and orange*
- 5.3. The backscattered amplitude map provided depth-dependent contrast and highlighted vessels based on their elevational position, useful for segmenting out-of-plane or low-amplitude vessels [1].
 - 5.3.1. LAB MEDIA: Figure 5. *Video editor: Highlight the rightmost full brain panel under "Backscattered Amplitude," then zoom in on the bottom right inset*
- 5.4. Vascular density maps acquired one week apart in the same mouse showed strong spatial overlap, confirming the reproducibility of coronal plane targeting using the Brain Positioning System [1].
 - 5.4.1. LAB MEDIA: Figure 6A. *Video editor: Highlight the "Comparison" image*
- 5.5. Quantitative analysis of the left somatosensory cortex across sessions showed similar distributions in vessel radius [1], velocity [2], and flow rate, demonstrating measurement reproducibility [3].
 - 5.5.1. LAB MEDIA: Figure 6B. *Video editor: Highlight the box plots labeled "Radius" under "Week 1" and "Week 2" in the right panel.*
 - 5.5.2. LAB MEDIA: Figure 6B. *Video editor: Highlight the box plots labeled "Velocity" under "Week 1" and "Week 2" in the right panel.*

5.5.3. LAB MEDIA: Figure 6B. *Video editor: Highlight the box plots labeled “Flowrate” under “Week 1” and “Week 2” in the right panel.*

5.6. Similar reproducibility was observed in the left thalamus region, with consistent values for vessel radius [1], velocity [2], and flow rate between Week 1 and Week 2 sessions [3].

5.6.1. LAB MEDIA: Figure 6C. *Video editor: Highlight the box plots labeled “Radius” under “Week 1” and “Week 2” in the right panel.*

5.6.2. LAB MEDIA: Figure 6C. *Video editor: Highlight the box plots labeled “Velocity” under “Week 1” and “Week 2” in the right panel.*

5.6.3. LAB MEDIA: Figure 6C. *Video editor: Highlight the box plots labeled “Flowrate” under “Week 1” and “Week 2” in the right panel.*

Pronunciation Guide:

1. Ultrasound

Pronunciation link:

<https://www.merriam-webster.com/dictionary/ultrasound>

IPA: /'ʌltrəˌsaʊnd/

Phonetic Spelling: uhl-truh-sound

2. Localization

Pronunciation link:

<https://www.merriam-webster.com/dictionary/localization>

IPA: /ˌləʊkəleɪˈzeɪʃən/

Phonetic Spelling: loh-kuh-luh-zay-shuhn

3. Microscopy

Pronunciation link:

<https://www.merriam-webster.com/dictionary/microscopy>

IPA: /maɪˈkrɑːskəpi/

Phonetic Spelling: my-krah-skuh-pee

4. Microvasculature

Pronunciation link:

<https://www.merriam-webster.com/dictionary/microvasculature>

IPA: /ˌmaɪkrəʊˈvæskjələr/

Phonetic Spelling: my-kroh-vas-kyuh-ler

5. Stereotaxic

Pronunciation link:

<https://www.merriam-webster.com/dictionary/stereotaxic>

IPA: /ˌstəriəʊˈtæksɪk/

Phonetic Spelling: stair-ee-oh-tak-sik

6. Isoflurane

Pronunciation link:

<https://www.merriam-webster.com/dictionary/isoflurane>

IPA: /ˌaɪsoʊˈflʊreɪn/

Phonetic Spelling: eye-soh-FLOOR-ane

7. Catheterization

Pronunciation link:

<https://www.merriam-webster.com/dictionary/catheterization>

IPA: /ˌkæθɪtərɪˈzeɪʃən/

Phonetic Spelling: kath-uh-ter-ih-zay-shuhn

8. Vasodilation

Pronunciation link:

<https://www.merriam-webster.com/dictionary/vasodilation>

IPA: /ˌveɪzəʊdɪˈleɪʃən/

Phonetic Spelling: vay-zoh-dye-lay-shuhn

9. Doppler

Pronunciation link:

<https://www.merriam-webster.com/dictionary/doppler>

IPA: /ˈdɑːplər/

Phonetic Spelling: dop-ler

10. Angio3D

Pronunciation link:

<https://www.howtopronounce.com/angio>

IPA: /ˈændʒioʊ θriː diː/

Phonetic Spelling: an-jee-oh three dee

11. Microbubble

Pronunciation link:

<https://www.merriam-webster.com/dictionary/microbubble>

IPA: /ˈmaɪkroʊˌbʌbəl/

Phonetic Spelling: my-kroh-buh-buhl

12. Coronal

Pronunciation link:

<https://www.merriam-webster.com/dictionary/coronal>

IPA: /kəˈroʊnəl/

Phonetic Spelling: kuh-roh-nuhl

13. Thalamus

Pronunciation link:

<https://www.merriam-webster.com/dictionary/thalamus>

IPA: /ˈθæləməs/

Phonetic Spelling: THAL-uh-muhs

14. Somatosensory

Pronunciation link:

<https://www.merriam-webster.com/dictionary/somatosensory>

IPA: /soʊˌmɛtəʊˈsɛnsəri/

Phonetic Spelling: soh-may-toh-SEN-suh-ree

15. Reproducibility

Pronunciation link:

<https://www.merriam-webster.com/dictionary/reproducibility>

IPA: /ˌrɪːprəˌduːsəˈbɪləti/

Phonetic Spelling: ree-pruh-doo-suh-BIL-uh-tee

16. Bifurcations

Pronunciation link:

<https://www.merriam-webster.com/dictionary/bifurcation>

IPA: /ˌbaɪfərˈkeɪʃən/

Phonetic Spelling: bye-fur-KAY-shuhn

17. Perfusion

Pronunciation link:

<https://www.merriam-webster.com/dictionary/perfusion>

IPA: /pərˈfjuːʒən/

Phonetic Spelling: per-FYOO-zuhn

18. Axial

Pronunciation link:

<https://www.merriam-webster.com/dictionary/axial>

IPA: /ˈæksjəl/

Phonetic Spelling: AK-shuhl

19. Elevational

Pronunciation link:

<https://www.merriam-webster.com/dictionary/elevational>

IPA: /ˌɛləˈveɪʃənəl/

Phonetic Spelling: el-uh-VAY-shuh-nuhl

20. Quantitative

Pronunciation link:

<https://www.merriam-webster.com/dictionary/quantitative>

IPA: /ˈkwɑːntəˌteɪtɪv/

Phonetic Spelling: KWAHN-tuh-tay-tiv