

Submission ID #: 70466

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Project Page Link: https://review.jove.com/files_upload.php?src=21492178

Title: The Assessment of Lymphatic Drainage Using ICG Near-Infrared Imaging and Ultrasound After Fracture

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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**

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. **Yes**

Current Protocol Length

Number of Steps: 34

Number of Shots: 59

Interviews

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

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Wenjing Zheng:** Our research evaluates lymphatic reflux after fractures using near-infrared imaging and B-type ultrasound to monitor drainage function and lymph node swelling.
 - 1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: 2.7.2*

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Wenjing Zheng:** Existing methods lack standardized dynamic in vivo assessment, so our protocol combines ICG-NIR imaging with ultrasound for reproducible monitoring.
 - 1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.2.1*

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Wenjing Zheng:** This protocol measures lymphatic drainage efficiency and popliteal lymph node enlargement after fracture in mice.
 - 1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Wenjing Zheng:** The key challenge is maintaining consistent imaging parameters, injection accuracy, and stable limb positioning during ultrasound scanning.

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

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Wenjing Zheng:** Future studies can combine immunological markers and histology to validate lymphatic mechanisms during fracture healing.

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

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

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

NOTE: The authors have not provided the title

What problem in your field of research does your JoVE article address?

- 1.6. **Wenjing Zheng, Longhua Hospital, Shanghai University of Traditional Chinese Medicine** (authors will present their testimonial statements live)

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

How does the video format of your article help you to share your work with the broader scientific community?

- 1.7. **Wenjing Zheng, Longhua Hospital, Shanghai University of Traditional Chinese Medicine** : (authors will present their testimonial statements live)

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

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee (IACUC) at Shanghai Model Organisms Center

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. Near-Infrared Indocyanine Green Imaging

Demonstrator: Yuecheng Cui

- 2.1. To begin, weigh 0.2 milligrams of indocyanine green or ICG (*I-C-G*) [1], transfer it to a 1.5-milliliter microcentrifuge tube [2], and dissolve it in 1 milliliter of PBS to prepare a 0.2-milligram per milliliter ICG solution [3]. Wrap the tube with aluminum foil to protect it from light [4].
 - 2.1.1. WIDE: Talent weighing 0.2 milligrams of indocyanine green. ***Videographer:** Please obtain CU shot as well for this shot.*
Videographer's NOTE: Use take 3 (clip A0003). Please use the last part of the clip, roughly from 00:22.
 - 2.1.2. Talent transferring indocyanine green to a 1.5-milliliter microcentrifuge tube.
 - 2.1.3. Talent dissolving indocyanine green in phosphate-buffered saline.
 - 2.1.4. Talent wrapping the microcentrifuge tube with aluminum foil.
- 2.2. Then, turn on the imaging system [1] and open FluoBeam (*Fluo-Beam*) version 1.46 [2]. Adjust the camera height as required [3].
 - 2.2.1. Talent turning on the imaging system.
 - 2.2.2. Show FluoBeam version 1.46 opening.
 - 2.2.3. Talent adjusting the camera height.
- 2.3. After anesthetizing the C57 male mice with a fracture model [1-TXT], moisten a cotton swab with 75% ethanol and disinfect the plantar surface of the modeled hind paw [2].
 - 2.3.1. Shot of the anaesthetized mice placed on the surgical platform. **TXT: Anesthesia: Induction: 2–3% Isoflurane; Maintenance: 1-2% Isoflurane**
 - 2.3.2. Talent moistening a cotton swab with 75% ethanol and wipes the hind paw
- 2.4. Using a 10-microliter microsyringe, aspirate 6 microliters of the ICG solution [1]. Insert the needle approximately 1 millimeter into the plantar surface and inject the solution at a constant rate until the full volume is delivered [2].

- 2.4.1. Talent aspirating 6 microliters of the indocyanine green solution into a 10-microliter microsyringe.
- 2.4.2. Talent inserting the microsyringe needle into the plantar surface and injecting ICG
- 2.5. In the software, activate both the **camera** and **laser** functions [1]. Click **modify** to create a new folder and set it as the save directory. Keep the exposure time identical for image acquisition at 0 and 24 hours [2].
 - 2.5.1. Show the camera and laser functions being activated in the software.
 - 2.5.2. Show clicking modify to create a new folder and set it as the save directory. Show the exposure time setting for the 0-hour and 24-hour image acquisitions.
- 2.6. Place the mouse under the camera in the prone position with the modeled hind limb centered in the field of view [1].
 - 2.6.1. Talent positioning the mouse under the camera in the prone position with the modeled hind limb centered in the field of view.
- 2.7. Set the exposure time to 30 milliseconds for both the 0- and 24-hour images [1]. Acquire images of the same plantar injection region of interest at 0 and 24 hours post-injection to measure ICG signal intensity [2].
 - 2.7.1. Show the exposure time being set to 30 milliseconds.
 - 2.7.2. Show image acquisition of the same plantar injection region of interest at 0 hours and 24 hours post-injection.
- 2.8. When the lymphatic vessels of the hind limb are clearly visible in the field of view, click **photo** to capture the images [1].
 - 2.8.1. Show the lymphatic vessels clearly visible in the field of view and click **photo** to capture the images.
- 2.9. After image acquisition, export the data [1].
 - 2.9.1. Show the acquired data being exported.
- 2.10. Open the image analysis software and import the fluorescence images by clicking **File** and selecting **Open** [1]. Open the 0-hour plantar image corresponding to the same animal and the same examination day [2].
 - 2.10.1. SCREEN: 70466-screenshot-1.mp4: 00:06-00:16
 - 2.10.2. SCREEN: 70466-screenshot-1.mp4: 00:16-00:20
- 2.11. Then, select **Oval**, click **Edit**, choose **Selection**, and select **Specify** [1]. Set the Width and Height to 30, then choose **Oval**, and click **OK** [2].
 - 2.11.1. SCREEN: 70466-screenshot-1.mp4: 00:25-00:35

- 2.11.2. SCREEN: 70466-screenshot-1.mp4: 00:35-00:40
- 2.12. Place the circle over a background area [1]. Click **Analyze** and select **Measure**. Record the Mean value in the Results window as fluorescence intensity at 0 hours background [2].
- 2.12.1. SCREEN: 70466-screenshot-1.mp4: 00:40-00:43
- 2.12.2. SCREEN: 70466-screenshot-1.mp4: 00:43-00:53
- 2.13. Then, place the circle over the target observation site and record the mean value as demonstrated earlier [1].
- 2.13.1. SCREEN: 70466-screenshot-1.mp4: 00:53-00:56
- 2.14. Calculate the corrected fluorescence intensity at 0 hours as FI0h (*F-I-Zero-Hour*) [1-TXT].
- 2.14.1. SCREEN: 70466-screenshot-1.mp4: 00:56-01:03, 01:16-01:23 **TXT:**
Corrected FI0h = FI0h – FI0hbg
- 2.15. Open the matched 24-hour image and repeat the previous steps to obtain the corrected fluorescence intensity at 24 hours.
- 2.15.1. SCREEN: 70466-screenshot-1.mp4: 01:27-01:29, 01:48-01:51, 02:15-02:18
- 2.16. Calculate the clearance rate using the background-corrected fluorescence intensity as presented here [1].
- 2.16.1. TEXT ON A PLAIN BACKGROUND
Clearance rate = $\frac{[(FI0h - FI0hbg) - (FI24h - FI24hbg)]}{(FI0h - FI0hbg)} \times 100\%$

3. Ultrasound Imaging and Lymph Node Delineation

Demonstrator: Yuecheng Cui

- 3.1. Turn on the ultrasound system [1], connect the transducer [2], and position the mouse so that the hind limb is clearly visualized [3]. Under continuous anesthesia, place the mouse in the prone position on the imaging stage with the head oriented away from the operator and the tail toward the operator [4]. Flex the knee so that the popliteal fossa faces upward and slightly laterally [5].
- 3.1.1. WIDE: Talent turning on the ultrasound system.
- 3.1.2. Talent connecting the transducer to the ultrasound system.
- 3.1.3. Talent positioning the mouse for clear visualization of the hind limb.
- 3.1.4. Talent placing the anesthetized mouse in the prone position on the imaging stage with the head oriented away from the operator and the tail

toward the operator.

- 3.1.5. Talent flexing the knee to orient the popliteal fossa upward and slightly laterally.
- 3.2. Now, position the transducer over the depression of the popliteal fossa [1]. Apply ultrasound coupling gel to the modeled hind limb [2] and rotate the transducer so that its angle remains parallel to the longitudinal axis of the hind limb [3].
 - 3.2.1. Talent positioning the transducer over the depression of the popliteal fossa.
 - 3.2.2. Talent applying ultrasound coupling gel to the modeled hind limb.
 - 3.2.3. Talent rotating the transducer parallel to the longitudinal axis of the hind limb.
- 3.3. Set the scan distance to 4 millimeters and the step size to 0.04 millimeters [1]. Identify the popliteal lymph node on the image according to its anatomical location [2].
 - 3.3.1. SCREEN: Show the scan distance set to 4 millimeters and the step size set to 0.04 millimeters on the ultrasound system.
 - 3.3.2. SCREEN: Show the popliteal lymph node identified on the ultrasound image according to its anatomical location.
- 3.4. Stabilize the mouse by holding the tail and modeled hind limb with both hands, avoiding any movement during transducer scanning [1].
 - 3.4.1. Talent stabilizing the mouse by holding the tail and the modeled hind limb with both hands.
- 3.5. Acquire images and identify the popliteal lymph node as a small oval or bean-shaped structure with intermediate-to-low echogenicity [2].
 - 3.5.1. SCREEN: Show the popliteal lymph node identified on the ultrasound image.
- 3.6. Export the images to the 3-dimensional reconstruction software for 3-dimensional image processing [1].
 - 3.6.1. SCREEN: Show the ultrasound images being exported to the 3-dimensional reconstruction software.
- 3.7. ~~After completing the 3 dimensional scan on the ultrasound imaging system console, save the scan as a 3 dimensional dataset [1].~~ Record the animal identification, group code, and time point at 0 hours or 24 hours [1].
 - 3.7.1. ~~Show the completed 3 dimensional scan being saved as a 3 dimensional dataset.~~ NOTE: This shot was not filmed.
 - 3.7.2. SCREEN: Show the animal identification, group code, and time point

information being recorded.

~~3.8. Export the raw data from the acquisition console to the analysis computer, preserving the original folder structure and using separate folders for each animal and time point whenever possible [1].~~

~~3.8.1. Show the raw data being exported from the acquisition console to the analysis computer. Show the different folders.~~ NOTE: This shot was not filmed.

3.9. Open the 3-dimensional reconstruction software. Click **Study** to enter the module and select **Import** [1].

3.9.1. SCREEN: Show the 3-dimensional reconstruction software opening. clicking Study and import to enter the module

3.10. Next, click the data folder, check the data to be imported, and double-click to open it [1].

3.10.1. SCREEN: Show the data folder being selected. The data selected for import. The selected data is being clicked.

3.11. In the display mode, select **Image Processing**, then choose **Load into 3D**, select **Transverse View**, choose **Volume Measurement**, and click **Start** [1]. Confirm that the slice orientation and step parameters are consistent with the acquisition settings [2].

3.11.1. SCREEN: Show the Image Processing option, Load into 3D selected in the display mode. Show the Transverse View, Volume Measurement and start option selected

3.11.2. SCREEN: Show the slice orientation and step parameters being checked against the acquisition settings.

3.12. Then, use the mouse wheel or slice slider to scroll from the proximal end to the distal end and identify the first slice in which the popliteal lymph node appears [1].

3.12.1. SCREEN: Show scrolling through image slices from the proximal end to the distal end and the first slice in which the popliteal lymph node appears

3.13. Begin tracing on the first slice in which the popliteal lymph node appears by left-clicking to start the contour [1]. Trace a closed contour point-by-point along the lymph node edge, keeping it close to the outer capsule and excluding surrounding fat and vascular lumen [2].

3.13.1. SCREEN: Show left-clicking to begin contour tracing on the first slice containing the popliteal lymph node.

3.13.2. SCREEN: Show contour tracing point by point along the edge of the lymph node and closely aligned to the outer capsule.

- 3.14. Use the mouse wheel to move to the slice showing the maximum cross-sectional area of the popliteal lymph node [1] and repeat the previous contour tracing step to complete the second contour [2].
 - 3.14.1. SCREEN: Show scrolling to the slice with the maximum cross-sectional area of the popliteal lymph node.
 - 3.14.2. SCREEN: Show the second contour traced around the popliteal lymph node.
- 3.15. Next, move to the slice just before the popliteal lymph node disappears and repeat the contour tracing step to complete the third contour [1]. Then, click **Finish** to generate the 3-dimensional segmented volume and output the volume measurement [2].
 - 3.15.1. SCREEN: Show scrolling to the slice just before the popliteal lymph node disappears and tracing the third contour
 - 3.15.2. SCREEN: Show clicking Finish to generate the 3-dimensional segmented volume. Show the generated volume measurement output.
- 3.16. Record the popliteal lymph node volume in cubic millimeters in the results panel [1].
 - 3.16.1. SCREEN: Show the popliteal lymph node volume displayed in the results panel.
- 3.17. In the measurement results panel, select **Export** and export the results as a .csv (C-S-V) or .xls (X-L-S) file [1]. Select the file type and export the image as TIFF Image Area [2].
 - 3.17.1. SCREEN: Show the Export option selected in the measurement results panel. Show the results exported as a .csv or .xls file.
 - 3.17.2. SCREEN: Show the file type selection menu and the image exported as TIFF Image Area .tif/
- 3.18. At the end of the experiment, place the mouse on a heating pad and allow it to recover from anesthesia [1].
 - 3.18.1. Talent placing the mouse on a heating pad.

Results

4. Results

- 4.1. On days 1, 7, and 14 after fracture model establishment, 24-hour plantar ICG fluorescence intensity was consistently higher in the fracture group than in the sham surgery group [1].
 - 4.1.1. LAB MEDIA: Figure 3. *Video editor: Highlight the plantar images in the "Fracture" row across 1 day, 7 day, and 14 day*
- 4.2. The lymphatic ICG clearance rate in the fracture group was significantly lower than that in the sham surgery group [1].
 - 4.2.1. LAB MEDIA: Figure 4. *Video editor: Highlight the red square groups below the blue circle groups at 1, 7, and 14 on the graph*
- 4.3. Popliteal lymph node volume in the fracture group mice was significantly higher than that in the control group on days 1, 7, and 14 [1].
 - 4.3.1. LAB MEDIA: Figure 5. *Video editor: Highlight the red square groups above the blue circle groups at 1, 7, and 14 on the graph*

1. Indocyanine
 Pronunciation link: <https://www.merriam-webster.com/dictionary/indocyanine>
 IPA: /ˌɪn.dooˈsaɪ.ə.niːn/
 Phonetic Spelling: in·doh·sy·uh·neen
2. Microcentrifuge
 Pronunciation link: <https://www.merriam-webster.com/dictionary/microcentrifuge>
 IPA: /ˌmaɪ.kroʊˈsen.trəˌfjuːdʒ/
 Phonetic Spelling: my·kroh·sen·truh·fyooj
3. Phosphate-buffered
 Pronunciation link: <https://www.merriam-webster.com/dictionary/phosphate>

- IPA: /'fɑ:s,fert/
Phonetic Spelling: faas·fayt
4. FluoBeam
Pronunciation link: No confirmed link found
IPA: /'flu:.oo,bi:m/
Phonetic Spelling: floo·oh·beem
 5. Anesthetizing
Pronunciation link: <https://www.merriam-webster.com/dictionary/anesthetize>
IPA: /ə'nesθə,təɪz/
Phonetic Spelling: uh·ness·thuh·tyz
 6. Isoflurane
Pronunciation link: <https://www.merriam-webster.com/medical/isoflurane>
IPA: /,aɪ.sou'flʊr,ɛn/
Phonetic Spelling: eye·soh·floor·ayn
 7. Microsyringe
Pronunciation link: <https://www.merriam-webster.com/dictionary/microsyringe>
IPA: /,maɪ.kroʊ.sə'rɪndʒ/
Phonetic Spelling: my·kroh·suh·rinj
 8. Fluorescence
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>
IPA: /flʊ'res.əns/
Phonetic Spelling: floor·ess·uhns
 9. Lymphatic
Pronunciation link: <https://www.merriam-webster.com/dictionary/lymphatic>
IPA: /lɪm'fæt.ɪk/
Phonetic Spelling: lim·fat·ik
 10. Ultrasound
Pronunciation link: <https://www.merriam-webster.com/dictionary/ultrasound>
IPA: /'ʌl.trə,saʊnd/
Phonetic Spelling: uhl·truh·sownd
 11. Transducer
Pronunciation link: <https://www.merriam-webster.com/dictionary/transducer>
IPA: /trænz'du:.sə/
Phonetic Spelling: tranz·doo·sur
 12. Popliteal
Pronunciation link: <https://www.merriam-webster.com/medical/popliteal>
IPA: /,pɑ:p.lə'ti:.əl/
Phonetic Spelling: pop·luh·tee·uhl
 13. Fossa
Pronunciation link: <https://www.merriam-webster.com/medical/fossa>
IPA: /'fɑ:.sə/
Phonetic Spelling: faa·suh
 14. Echogenicity
Pronunciation link: <https://www.howtopronounce.com/echogenicity>

- IPA: /ˌɛk.oʊ.dʒəˈnɪs.ə.ti/
Phonetic Spelling: ek·oh·juh·niss·uh·tee
15. Reconstruction
Pronunciation link: <https://www.merriam-webster.com/dictionary/reconstruction>
IPA: /ˌriː.kənˈstrʌk.tʃən/
Phonetic Spelling: ree·kuhn·struk·shuhn
16. Transverse
Pronunciation link: <https://www.merriam-webster.com/dictionary/transverse>
IPA: /trænzˈvɜːs/
Phonetic Spelling: tranz·vurs
17. Segmented
Pronunciation link: <https://www.merriam-webster.com/dictionary/segmented>
IPA: /ˈseg.men.tɪd/
Phonetic Spelling: seg·men·tid
18. Cubic
Pronunciation link: <https://www.merriam-webster.com/dictionary/cubic>
IPA: /ˈkjuː.bɪk/
Phonetic Spelling: kyoo·bik
19. Anesthesia
Pronunciation link: <https://www.merriam-webster.com/dictionary/anesthesia>
IPA: /ˌæn.əsˈθiː.zə/
Phonetic Spelling: an·uhs·thee·zhuh
20. Ethanol
Pronunciation link: <https://www.merriam-webster.com/dictionary/ethanol>
IPA: /ˈeθ.əˌnɑːl/
Phonetic Spelling: eth·uh·naal
21. Contour
Pronunciation link: <https://www.merriam-webster.com/dictionary/contour>
IPA: /ˈkɑːn.tɔr/
Phonetic Spelling: kaan·toor
22. Longitudinal
Pronunciation link: <https://www.merriam-webster.com/dictionary/longitudinal>
IPA: /ˌlɑːn.dʒəˈtuː.dən.əl/
Phonetic Spelling: laan·juh·too·duh·nuhl
23. Capsule
Pronunciation link: <https://www.merriam-webster.com/dictionary/capsule>
IPA: /ˈkæp.suːl/
Phonetic Spelling: kap·sool
24. Vascular
Pronunciation link: <https://www.merriam-webster.com/dictionary/vascular>
IPA: /ˈvæs.kjə.lə/
Phonetic Spelling: vas·kyuh·lur
25. Fluorescent
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescent>

IPA: /floʻres.ənt/

Phonetic Spelling: floor·ess·uhnt