

Submission ID #: 70088

Scriptwriter Name: Sulakshana Karkala

Project Page Link: <https://review.jove.com/account/file-uploader?src=21376888>

Title: Preparation and Live Imaging of Regenerating *Hydra* Spheroids

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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? **Yes**

Microscope: Leica M80

SCOPE: 2.1.2, 2.2.1, 2.3.1, 2.3.2, 2.3.3, 2.5.1, 2.5.2, 2.10.1, 2.10.2

***Videographer:** Please capture all shots labeled SCOPE with a SCOPE kit*

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? **Yes**

If **Yes**, how far apart are the locations? **2.5km**

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: 19

Number of Shots: 41

Introduction

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. **Sera Weevers:** My research involves investigating the mechanisms of symmetry breaking in regenerating Hydra spheroids
 - 1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~**Describe the research contexts or systems in which this protocol can be applied.**~~

- 1.2. **Sera Weevers:** This protocol is broadly applicable to other model systems that rely on long-term imaging to study dynamic morphogenetic processes.
 - 1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

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

- 1.3. **Sera Weevers:** These microscopy techniques allow researchers to create long-term live imaging datasets without photodamaging their samples.
 - 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. **Sera Weevers:** It is important to choose the most applicable mounting and microscopy methods for the intended results.
 - 1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera

~~Are there any additional methods or analyses that can be performed following this procedure?~~

1.5. **Sera Weevers:** Once imaged, the data should be analyzed properly. There are many image analysis softwares and techniques that can be used.

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.

Protocol

~~2. Generation and Long Term Live Imaging of Hydra Tissue Spheroids~~

Generation of *Hydra* Tissue Spheroids

Demonstrator: Sera Weevers

2.1. To begin, using a glass Pasteur pipette, move a *Hydra* to a lid of a 10-centimeter culture dish [1]. Position it under a stereoscope [2].

2.1.1. WIDE: Talent using a glass Pasteur pipette to transfer a *Hydra* onto the lid and placing the lid under the stereoscope.

2.1.2. SCOPE: View of the *Hydra* through the microscope.

Videographer: Please capture all shots labeled SCOPE with a SCOPE kit

2.2. Bisect the *Hydra* by creating a single, straight cut between body regions B2 and B3 [1].

2.2.1. SCOPE: Talent making a single straight cut through the *Hydra* between body regions B2 and B3.

2.3. Allow the bisected tissue to relax until the cut end remains swollen [1]. Make a second cut where the swelling ends to generate a ring of tissue [2]. Generate tissue rings from each of the *Hydra* halves [3].

2.3.1. SCOPE: Shot of the bisected *Hydra* tissue relaxing and the cut end swelling.

2.3.2. SCOPE: Talent making a second cut at the end of the swollen region to form a tissue ring.

2.3.3. SCOPE: Talent repeating the cutting process to obtain tissue rings from both *Hydra* halves.

2.4. Remove the remaining head and foot ends from the lid [1].

2.4.1. Talent removing the discarded head and foot tissue pieces from the lid.

2.5. Then cut the tissue rings open [1]. Cut the opened rings into multiple rectangular tissue pieces [2-TXT].

2.5.1. SCOPE: Talent cutting each tissue ring open.

2.5.2. SCOPE: Talent cutting the opened rings into multiple smaller tissue pieces. **TXT:**
Size dimension: 100 - 150 μ m by 300 - 500 μ m

- 2.6. Incubate the tissue pieces in room-temperature dissociation medium for 3 to 4 hours [1].
 - 2.6.1. Talent transferring the tissue pieces into a container holding room-temperature DM.

Mounting *Hydra* Spheroids for Imaging

NOTE: Treat this as a separate section

- 2.7. To mount the spheroids in agarose, first completely coat the bottom of a 4-well glass-bottom imaging plate with a 2-millimeter-thick layer of 1% agarose [1]. When the agarose has solidified [2], push the tip of a P1000 (*P-one-thousand*) pipette into the agarose until it touches the bottom of the imaging plate [3].
 - 2.7.1. Talent pouring liquid agarose into a 4-well glass-bottom imaging plate to coat the bottom evenly.
 - 2.7.2. Shot of the solidified agarose.
 - 2.7.3. Shot of a P1000 pipette being pushed into the agarose.
- 2.8. Hold the pipette straight and flush with the bottom [1], then wiggle and rotate the tip to cut the agarose [2]. Release the plunger and slowly remove the pipette tip straight up [3].
 - 2.8.1. Shot of the pipette being held straight.
 - 2.8.2. Talent wiggling and rotating the pipette tip to cut the agarose.
 - 2.8.3. Talent releasing the plunger and slowly removing the pipette tip vertically.
- 2.9. Repeat the gel-cutting process until enough wells have been created [1]. Then cover the agarose with a thin layer of *Hydra* medium [2]. Release any air bubbles in the agarose wells by touching them with a P20 pipette tip [3].
 - 2.9.1. Shot of wells on the plate.
 - 2.9.2. Talent gently adding a thin layer of HM over the agarose.
 - 2.9.3. Talent using a P20 pipette tip to remove air bubbles from the wells.
- 2.10. After incubation, assess whether the spheroids have properly folded by checking for a rounded shape and complete ectoderm coverage [1]. Select folded spheroids with a diameter of 250 to 350 micrometers [2].
 - 2.10.1. SCOPE: Talent inspecting spheroids under a microscope to assess shape and ectoderm coverage.
 - 2.10.2. SCOPE: Talent selecting spheroids of the appropriate diameter.

- 2.11. Move the *Hydra* spheroids one by one into the imaging plate [1]. If a spheroid does not immediately drop into a well, gently nudge it toward a well using the pipette [2].
- 2.11.1. Talent transferring individual *Hydra* spheroids into the imaging plate.
- 2.11.2. Talent gently nudging a spheroid into an agarose well with a pipette.
- 2.12. To mount in FEP (*F-E-P*), first fill the chamber with *Hydra* medium [1-TXT]. Release air bubbles from the wells using prepared glass pipettes with a scooping motion [2].
- 2.12.1. Talent filling the FEP chamber with HM. **TXT: FEP: Fluoroethylene propylene**
- 2.12.2. Talent using a glass pipette to scoop and remove air bubbles from FEP wells.
- 2.13. Once all wells are free of bubbles, move the *Hydra* spheroids one by one into the FEP chamber [1]. Nudge spheroids into wells with a pipette if needed [2].
- 2.13.1. Talent transferring *Hydra* spheroids into the FEP chamber.
- 2.13.2. Talent gently guiding a spheroid into a well using a pipette.

Long-Term Live Imaging of *Hydra* Spheroids

NOTE: Treat this as a separate section

- 2.14. Image the samples on an inverted microscope outfitted with a spinning disk unit [1]. Set the microscope chamber temperature to 18 to 21 degrees Celsius [2].
- 2.14.1. Talent operating an inverted spinning disk confocal microscope.
- 2.14.2. Display the temperature control unit showing the chamber temperature set between 18 and 21 degrees Celsius.
- 2.15. Choose an objective with a field of view that covers the complete sample [1]. Then place the sample holder into the microscope stage [2]. Turn on transmitted light and set the exposure time [3]. Select the laser wavelength, laser power, and exposure time for the fluorophores present [4].
- 2.15.1. Talent placing the sample holder onto the microscope stage.
- 2.15.2. SCREEN: 2.15.2. 00:10-00:20 **Author's NOTE:** Screen recordings may not be of the best quality. Our system is limited and we tried several times with our IT team to improve the screen recording quality but this is the best we can provide as we are not authorized to install any new softwares
- 2.15.3. SCREEN: 2.15.3.
- 2.15.4. SCREEN: 2.15.4. 00:04-00:11 and 00:23-00:26

2.16. Find all samples and define them as multiple imaging positions [1]. For each sample, create a z-stack from the plane closest to the objective to the first out-of-focus plane [2]. Set time-lapse parameters with an interval of 10 minutes and start imaging [3].

2.16.1. SCREEN: 2.16.1 00:02-00:12

2.16.2. SCREEN: 2.16.2 00:09-00:30.

2.16.3. SCREEN: 2.16.3 00:00-00:06 and 00:20-00:23.

2.17. Alternatively, image the samples on a light-sheet microscope with horizontally positioned detection objectives [1-TXT].

2.17.1. Talent operating a light-sheet fluorescence microscope. **TXT: Set chamber temperature to 18 - 21°C or cool the room instead**

2.18. Place the sample holder into the microscope [2].

2.18.1. Talent placing the sample holder into the light-sheet microscope.

2.19. Set all the required parameters and start imaging [3].

2.19.1. SCREEN: 2.19.3.

Results

3. Results

- 3.1. Long-term time-lapse datasets of regenerating *Hydra* spheroids were successfully generated using spinning disk confocal microscopy and light-sheet fluorescence microscopy [1].
 - 3.1.1. LAB MEDIA: Figure 5A, Figure 6A. *Video editor: Sequentially show 5 A and then 6 A*
- 3.2. In both imaging setups, spheroids broke symmetry, elongated, and developed tentacles and a foot within 3 days [1]. After prolonged imaging, regenerated animals became highly motile and moved partially or completely out of the field of view [2].
 - 3.2.1. LAB MEDIA: Supplementary video 2
 - 3.2.2. LAB MEDIA: Supplementary video 1
- 3.3. Using fluorescent markers, rapid reorganization of actin fibers was observed during *Hydra* spheroid regeneration [1]. Although only half of the spheroid was imaged, the region of interest containing the actin reorganization was successfully captured [2].
 - 3.3.1. LAB MEDIA: Figure 5B. *Video editor: Sequentially show images from left to right*
 - 3.3.2. LAB MEDIA: Figure 5C.
- 3.4. Organizer formation was visualized by imaging β -catenin-green fluorescent protein-expressing spheroids with light-sheet fluorescence microscopy [1].
 - 3.4.1. LAB MEDIA: Figure 6B. *Video editor: Sequentially show images from left to right*
- 3.5. Three stages of regeneration were resolved, including elevated nuclear β -catenin across the spheroid [1], localized nuclear β -catenin enrichment [2], and subsequent axis establishment with tentacle outgrowth [3].
 - 3.5.1. LAB MEDIA: Figure 6B. *Video editor: Highlight the image labeled 1 h*
 - 3.5.2. LAB MEDIA: Figure 6B. *Video editor: Highlight the image labeled 15 h*
 - 3.5.3. LAB MEDIA: Figure 6B. *Video editor: Highlight the image labeled 26 h*
- 3.6. Fusion of the two opposing views acquired using light-sheet fluorescence microscopy produced a dataset spanning the entire spheroid volume without apparent loss of image quality [1].
 - 3.6.1. LAB MEDIA: Figure 6C.

1. Hydra

Pronunciation link: <https://www.merriam-webster.com/dictionary/Hydra>

IPA: /'haɪ.drə/

Phonetic Spelling: hye·druh

2. Stereoscope

Pronunciation link: <https://www.merriam-webster.com/dictionary/stereoscope>

IPA: /'ster.i.ə.skəʊp/, /'sti:r.i.ə.skəʊp/

Phonetic Spelling: steh·ree·uh·skohp

3. Bisect

Pronunciation link: <https://www.merriam-webster.com/dictionary/bisect>

IPA: /baɪ'sekt/

Phonetic Spelling: bye·sekt

4. Ectoderm

Pronunciation link: <https://www.merriam-webster.com/dictionary/ectoderm>

IPA: /'ek.tə.də:m/

Phonetic Spelling: ek·tuh·durm

5. Spheroid

Pronunciation link: <https://www.merriam-webster.com/dictionary/spheroid>

IPA: /'sfɪr.ɔɪd/

Phonetic Spelling: sfeer·oyd

6. Agarose

Pronunciation link: <https://www.merriam-webster.com/dictionary/agarose>

IPA: /'æg.ə.roʊs/

Phonetic Spelling: ag·uh·rohs

7. Micrometer

Pronunciation link: <https://www.merriam-webster.com/dictionary/micrometer>

IPA: /maɪ'krɑː.mə.t̬ər/

Phonetic Spelling: my·krah·muh·tur

8. Fluoroethylene

Pronunciation link:

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

IPA: /ˌflʊr.oʊˈeθ.ə.liːn/

Phonetic Spelling: floor·oh·eth·uh·leen

9. Propylene

Pronunciation link: <https://www.merriam-webster.com/dictionary/propylene>

IPA: /ˈprɒp.ə.liːn/

Phonetic Spelling: proh·puh·leen

10. Confocal

Pronunciation link: <https://www.merriam-webster.com/dictionary/confocal>

IPA: /kənˈfəʊ.kəl/

Phonetic Spelling: kon·foh·kuhl

11. Fluorophore

Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorophore>

IPA: /ˈflʊr.əˌfɔːr/

Phonetic Spelling: floor·uh·for

12. Fluorescence

Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>

IPA: /flʊˈres.əns/, /flʊːˈres.əns/

Phonetic Spelling: floor·ess·uhns

13. Reorganization

Pronunciation link: <https://www.merriam-webster.com/dictionary/reorganization>

IPA: /ˌriː.ɔːr.gə.nəˈzeɪ.ʃən/

Phonetic Spelling: ree·or·guh·nuh·zay·shuhn

14. Regeneration

Pronunciation link: <https://www.merriam-webster.com/dictionary/regeneration>

IPA: /rɪˌdʒen.əˈreɪ.ʃən/

Phonetic Spelling: ree-jen-uh-ray-shuhn

15. β -catenin

Pronunciation link: <https://www.howtopronounce.com/%CE%B2-catenin>

IPA: /ˈbeɪ.tə kəˈtiː.nɪn/

Phonetic Spelling: bay-tuh kuh-tee-nin

16. Microscopy

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

IPA: /maɪˈkrɑː.skə.pi/

Phonetic Spelling: my-krah-skuh-pee

17. Tentacles

Pronunciation link: <https://www.merriam-webster.com/dictionary/tentacle>

IPA: /ˈten.tə.kəlz/

Phonetic Spelling: ten-tuh-kuhlz

18. Inverted

Pronunciation link: <https://www.merriam-webster.com/dictionary/inverted>

IPA: /ɪnˈvɜː.təd/

Phonetic Spelling: in-vur-tuhd

19. Dissociation

Pronunciation link: <https://www.merriam-webster.com/dictionary/dissociation>

IPA: /dɪˌsoʊ.siˈeɪ.ʃən/

Phonetic Spelling: dih-soh-see-ay-shuhn

20. Videographer

Pronunciation link: <https://www.merriam-webster.com/dictionary/videographer>

IPA: /ˌvɪd.iˈɑː.grə.fə/

Phonetic Spelling: ʒoʒ·ee·ah·gruh·fur