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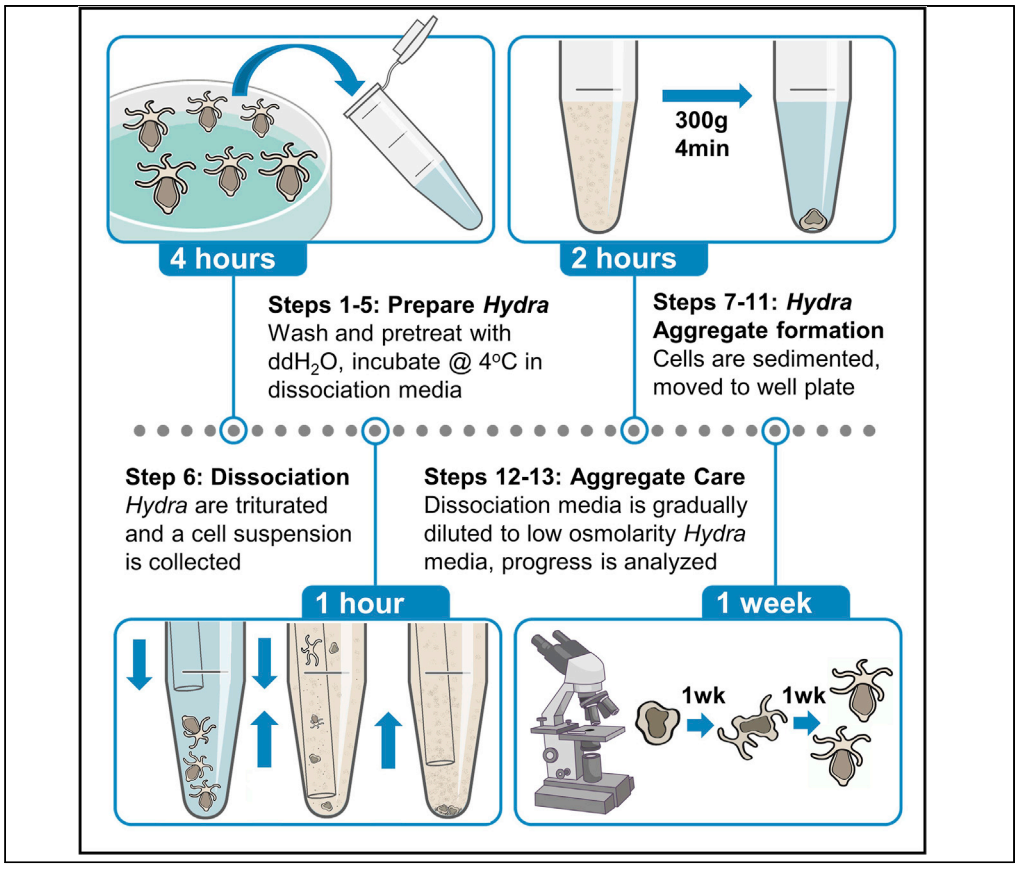
Dissociation and reaggregation of *Hydra vulgaris* for studies of self-organization



The remarkable regenerative abilities of the small cnidarian *Hydra vulgaris* include the capacity to reassemble itself after dissociation into individual cells. Here, we present a robust protocol for the dissociation and reaggregation of *Hydra* tissue that addresses many common challenges encountered during the preparation and execution of the dissociation, as well as the formation and care of regenerating cellular aggregates. Analysis of the process provides insight into the mechanisms of nervous system self-organization.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Amplify experimental *Hydra* and prepare for the animal's dissociation

Dissociate *Hydra* into individual cells, aggregate, and monitor their reassembly

Follow *Hydra's* epithelial layer segregation and neural circuit formation

A new polyp emerges from the cellular aggregate after one week of development

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Dissociation and reaggregation of *Hydra vulgaris* for studies of self-organization

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SUMMARY

The remarkable regenerative abilities of the small cnidarian *Hydra vulgaris* include the capacity to reassemble itself after dissociation into individual cells. Here, we present a robust protocol for the dissociation and reaggregation of *Hydra* tissue that addresses many common challenges encountered during the preparation and execution of the dissociation, as well as the formation and care of regenerating cellular aggregates. Analysis of the process provides insight into the mechanisms of nervous system self-organization.

For complete details on the use and execution of this protocol, please refer to Lovas and Yuste (2021).

BEFORE YOU BEGIN

Amplify experimental *Hydra* on an every-other-day feeding schedule

Timing: 1–4 weeks

1. Expand *Hydra* colony by feeding on an approximately every-other-day schedule to numbers appropriate to the numbers of *Hydra* used in individual experiments and the frequency of experiments.
   a. *Hydra* should be starved at least 36 h and no more than 96 h before the start of the experiment.
      i. Times shorter than this can lead to incorporation of contents of the gut into aggregates.
      ii. Longer starvation periods lead to less efficient dissociation. Troubleshooting 1.

Note: The protocol described here calls for 50–70 individual *Hydra*. On an every-other-day feeding schedule the *Hydra* population will double every ~3–4 days depending on the strain. If multiple experiments are planned in succession (e.g., every-other-day), be sure to account for this and maintain a population of at least ~200 *Hydra* so the colony is not decimated. This number of animals fits well in a 15 cm tissue culture dish.

2. Thoroughly clean *Hydra* culture dishes at least one week before experiments (Figure 1A).

Note: *Hydra* have a naturally rich microbiome, and some level of microorganism growth in culture dishes is expected. While usually not a problem, the dissociation protocol disrupts *Hydra's* natural barrier against these microorganisms, so early efforts to minimize contamination of the preparation must be made in addition to the washing steps before dissociation and

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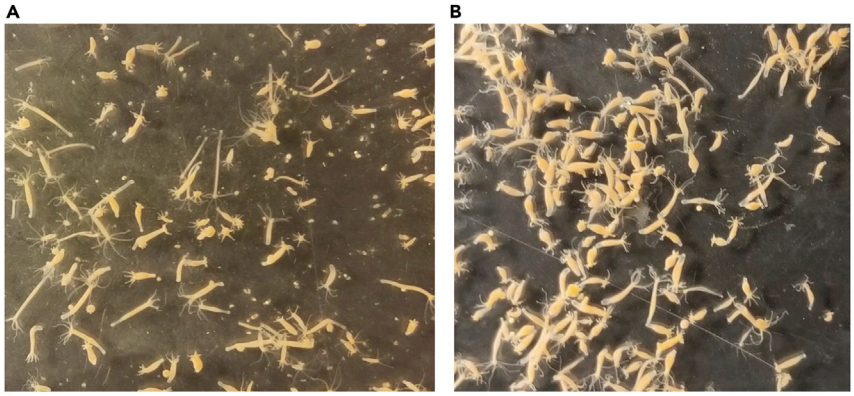

Figure 1. *Hydra* cleanliness and health

(A) *Hydra* 12 h post-feeding in an unwashed dish (neglected ~3 weeks). Note the digested food pellets and algal growth after 3 weeks due to the 12-h circadian light cycles of the incubator. *Hydra* should be detached and their dish thoroughly scrubbed at least once a week during experiments. Vigorous cleaning of digested food pellets and stray shrimp the day after feeding will also improve health.

(B) *Hydra* are immediately healthier with a cleaning of their dish. Tentacle length is one of the best indicators of animal health.

antibiotic treatment of dissociation medium with Penicillin/Streptomycin (100 U/mL). Troubleshooting 2.

⚠ CRITICAL: *Hydra* will also grow much faster in clean dishes. A neglected and recently cleaned dish are show in Figures 1A and 1B, respectively. Neglecting cleaning of the dish surface is a common mistake for novice *Hydra* culturists. Digested food pellets and debris are removed, and the dish is scrubbed clean with a kimwipe, rinsed, and *Hydra* are replaced. Troubleshooting 2.

Prepare dissociation medium and experiment supplies

Timing: 1–4 h

3. Prepare dissociation media from stock 20× concentrates of individual media components as described in the materials and equipment section.
   a. Adjust the pH of the 20× stock solutions of HEPES and Sodium Citrate to 7 to remove the need to adjust the pH of individual preparations of working concentration dissociation media. Later addition of Penicillin-Streptomycin concentrate to the working concentration dissociation media has a negligible effect on solution pH. Troubleshooting 3.
   b. After preparation, filter dissociation media through a 0.2 μm syringe filter before immediate use or storage. Troubleshooting 2.

Note: 200 mL dissociation media typically provides enough volume for 6 experiments, at roughly 33 mL/experiment, depending on the size and number of aggregates. Dissociation media can be frozen along with 20× stock solutions for future use for at least 6 months with no notable adverse effect on aggregate health.

⚠ CRITICAL: If any solutions are thawed during the process, either 20× stock concentrates or the working concentration dissociation media, inspect for precipitation of salts upon thawing. Vortex as needed until dissolved. Troubleshooting 3.

Figure 2. *Hydra* collected in a 3.5 cm dish for cleaning pre-dissociation

~70 *Hydra* collected from a clean dish. Note that even when collected from a clean dish some debris will likely still be present. This includes digested food pellets, pieces of cuticle/peduncle, and basal disk adhesive agitated during the transfer (white arrows), potentially curly shards of highly-autofluorescent plastic (red arrow) liberated when scraping the Pasteur pipette across the bottom of the dish to detach *Hydra* for the transfer. This debris should be completely removed, as anything present in the cell suspension will aggregate along with *Hydra* cells.

4. Prepare supplies and working area for the experiment (Figure 3).
   a. Supplies for initial cleaning, washing, and dissociation.
      i. Prepare an ice bin with a large working surface area.
      ii. Pre-chill two 1.5 mL microcentrifuge tubes on ice (one for the initial incubation, the other to collect the cell suspension after dissociation).
      iii. Keep the cell counting chamber/hemocytometer ready to count dissociated cells.
      iv. Prepare a 6-well plate or 3.5 cm dish to collect *Hydra* for cleaning.
   b. Bring 150 mL ddH_2O and 33 mL dissociation media to 0°C, place on ice to maintain temperature.
      i. Use a glass 250 mL Erlenmeyer flask to cool the ddH_2O rapidly. Thaw dissociation media in a 500 mL graduated cylinder filled with a bit of warm water or prepare fresh from concentrated 20× stock solutions. Check any thawed tubes for precipitate and vortex to re-dissolve as needed. Place on ice to bring to 0°C. Troubleshooting 3.
   c. Supplies for cell sedimentation and aggregate incubation.
      i. Pre-chill a number of 0.5 mL microcentrifuge tubes – according to approximately the number and size of aggregates desired – in the detached holder of a repurposed 1 mL pipette (P1000) tip rack.

Note: This rack size comfortably holds the 0.5 mL tubes and allows contact with ice when the rack is removed from the box, as well as easy transport and containment in future applications during the experiment.

      ii. Prepare a 24-well plate to hold aggregates after sedimentation.

⚠ CRITICAL: Be sure dissociation media is at pH7 and be sure *Hydra* cultures are clean (Figure 1). Be sure animals are starved at least 36 h. Also, when thawing any solution, thaw completely and vortex any precipitate back into solution.

Figure 3. *Hydra* dissociation materials

(A) 1 mL (P1000) pipette tip box repurposed as a 0.5 mL microcentrifuge tube holder. The detachable rack allows the pre-chilling of tubes before *Hydra* cell suspension is added.

(B) 6-well plate with experimental animals in *Hydra* media in the top-left well. The washes with *Hydra* media and ice-cold ddH_2O will be conducted here.

(C) Thawing working-concentration dissociation media prepared from 20× stock solutions. Be sure to check for precipitated salts!

(D) 24-well plate that will hold 600 µL dissociation media to pre-cool to 4°C, and eventually the aggregates.

(E) Two clean Pasteur pipettes, one fresh from the box for the actual mechanical trituration to dissociate the *Hydra*.

(F) Two pre-chilled 1.5 mL microcentrifuge tubes, one to hold *Hydra* during the 4°C incubation in dissociation medium and during the dissociation, and another to collect the cell suspension after sedimentation of undissociated tissue chunks.

(G) Hemocytometer to determine the concentration of cells in suspension.

(H) Ice bucket with a fairly large working surface area.

(I) ~150 mL ddH_2O chilling to 0°C in a glass Erlenmeyer flask, eventually used to wash *Hydra* before resuspension in dissociation media.

(J) Ice, enough to work for 3–4 h without melting, and with enough surface area to pre-chill the 0.5 mL tubes in the 1 mL tip rack. Refill if needed. Not pictured: *Hydra* media squirt bottle for the initial washes.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
$CaCl_2$-$2H_2O$	Sigma-Aldrich	Cat#C3881
$MgSO_4$-$7H_2O$	Sigma-Aldrich	Cat#M1880
KCl	Sigma-Aldrich	Cat#P9541
HEPES	Sigma-Aldrich	Cat#H3375
Na_2HPO_4	Sigma-Aldrich	Cat#S0876
KH_2PO_4	Sigma-Aldrich	Cat#P3786
Na Pyruvate	Sigma-Aldrich	Cat#P5280
Na_3 Citrate-$2H_2O$	Sigma-Aldrich	Cat#W302600

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Penicillin-Streptomycin 10,000 U/mL (100×)	Sigma-Aldrich	Cat#P0781
Other		
60 mL Syringe	BD	Cat#309653
0.20 µm Filter	Corning Incorporated	Cat#431219
50 mL Centrifuge Tubes	Corning Incorporated	Cat#430829
1.5 mL Microcentrifuge Tubes	Eppendorf	Cat#022363212
0.5 mL Microcentrifuge Tubes	Eppendorf	Cat#022363719
150 mm Borosilicate Type I Glass Pasteur Pipette	Wheaton Science Products	Cat#W357331
Rubber Pipette Bulb	Fisher Scientific	Cat#03-448-21
24-Well Tissue Culture Plate	Corning Incorporated	Cat#3527
6-Well Tissue Culture Plate	Corning Incorporated	Cat#3506
250 mL Erlenmeyer Flask	Pyrex	Cat#4980250
500 mL Graduated Cylinder	Pyrex	Cat#1060500
Bright Line Hemocytometer	Fisher Scientific	Cat#02-671-5
Hydra media squirt bottle	Thermo Scientific	Cat#24020500
Ice bucket	n/a	n/a
Centrifuge	n/a	n/a
P1000 Tip Box with Removable Rack	USA Scientific	Cat#1111-2821
Experimental models: Organisms/strains		
Hydra vulgaris (interstitial GCaMP6s transgenic)	*Hydra vulgaris* AEP strain	Dupre and Yuste, 2017

MATERIALS AND EQUIPMENT

Dissociation Medium		
Reagent	Final concentration	Amount
$CaCl_2$-$2H_2O$ (100 mM)	5 mM	10 mL
$MgSO_4$-$7H_2O$ (20 mM)	1 mM	10 mL
KCl (56 mM)	2.8 mM	10 mL
HEPES (220 mM)	11 mM	10 mL
Na_2HPO_4 (13.4 mM)	0.67 mM	10 mL
KH_2PO_4 (8.8 mM)	0.44 mM	10 mL
Na Pyruvate (100 mM)	5 mM	10 mL
Na_3 Citrate-$2H_2O$ (100 mM)	5 mM	10 mL
ddH_2O	n/a	118 mL
Penicillin/Streptomycin (10,000 U/mL)	100 U/mL	2 mL
Total	**n/a**	**200 mL**

Store stock solutions and working concentration dissociation media at -20°C. Store stock solutions in 8 separate centrifuge tubes. Solutions are stable for at least 6 months with freezing. To prepare working concentration dissociation media, combine the 8 chemicals each at 1/20th the final volume and bring the remaining 12/20 volume to 20/20 with water and Penicillin/Streptomycin (e.g., for 200 mL dissociation media, combine 10 mL from each of the stock solutions to a flask, and add 118 mL distilled water and 2 mL Penicillin/Streptomycin). Filter sterilize working concentration dissociation media before use or storage. Note, storage at -20°C can cause some salts to precipitate. Thaw solutions completely and vortex if necessary before any use. To obtain 20× concentrate stocks of individual chemicals, add to 100 mL ddH_2O in separate tubes: 1.47 g $CaCl_2$-$2H_2O$, 493 mg $MgSO_4$-$7H_2O$, 417 mg KCl, 5.48 g HEPES, 190 mg Na_2HPO_4, 119 mg KH_2PO_4, 1.1 g Na Pyruvate, 2.941 g Na_3 Citrate-$2H_2O$.

Alternatives: Minor variations in dissociation medium exist from protocol to protocol. One variation substitutes HEPES for TES and calls for the addition of glucose (Soriano et al.,

2009; Cochet-Escartin et al., 2017; Hobmayer et al., 2001). Many substitute the 100 U/mL penicillin/streptomycin for rifampicin at 50 μg/mL (Soriano et al., 2009; Skokan et al., 2020; Cochet-Escartin et al., 2017; Hobmayer et al., 2001).

STEP-BY-STEP METHOD DETAILS

Prepare *Hydra* for incubation in dissociation medium

Timing: ~4 h

Experimental animals are collected, thoroughly washed in *Hydra* media and ice-cold ddH_2O, resuspended in ice-cold relatively isosmotic dissociation media, and then incubated at 4°C before dissociation. Methods Videos S1 and S2 accompany this part of the protocol.

1. Collect 50–70 non-budding *Hydra* (Figure 2).
   a. Using an unpolished Pasteur pipette, collect *Hydra* in a 3.5 cm dish or one well of a 6-well plate. *Hydra* can be detached with gentle pipette aspiration and tend to settle and reattach rapidly once fluid flow has stopped (including reattaching inside the pipette, if the transfer is not immediate).
   b. Sizes should be roughly representative of the distribution of sizes in the parent dish.
   c. Budding *Hydra* can be used with no adverse effects on experimental outcome, but are generally excluded to maintain a faster rate of colony growth.

   Note: This protocol is optimized for 50–70 *Hydra*. With lower densities the dissociation will be more difficult, and with higher densities a larger proportion of 'clumps' or chunks of tissue following dissociation complicates the procedure. Troubleshooting 1 and 3.

   Pause point: Provided they haven't been fed within the last 24 h, collected *Hydra* can remain in their new dish another 24 h before proceeding to step 2.

2. Wash *Hydra* 5× with 21°C–25°C *Hydra* media (Methods Video S1).
   a. Remove ~75%–90% of the original *Hydra* media with a clean 15 cm Pasteur pipette.
   b. Using a *Hydra* media squirt bottle, aggressively spray *Hydra* feet attached to the near-empty dish with the goal of detaching as many *Hydra* from the surface as possible.
   c. As the well of the dish begins to fill, angle and stabilize the stream of the bottle to form a whirlpool to collect the *Hydra* in the middle of the dish to make media removal easier.
   d. Collect and dispose of as much floating debris as possible while the *Hydra* are grouped in the middle of the dish after the whirlpool. Troubleshooting 2.

   Note: Hydra foot substrate attachment typically involves secretion of adhesive compounds (Rodrigues et al., 2016), and bacterial biofilms have a tendency to form around the feet (Augustin et al., 2017). As much of this debris as possible should be removed. In rare cases, during this step a tentacle or two or pieces of *Hydra* cuticle may detach, and should be removed as well.

   ⚠ CRITICAL: In addition, scraping the plastic bottom of any culture dishes with the Pasteur pipette during *Hydra* transfer can shred pieces of highly-autofluorescent plastic into the media, which will reaggregate with *Hydra* tissue if not removed (Figure 2).

   e. Repeat the process 4 times until *Hydra* are thoroughly cleaned and debris is removed.

   Optional: Repeat wash steps until all debris is removed from media.

3. Wash *Hydra* 4× with 0°C ddH_2O (Methods Video S2).
   a. Use a glass 250 mL Erlenmeyer flask to cool the ddH_2O rapidly. Thaw dissociation media in a 500 mL graduated cylinder filled with a bit of warm water or prepare fresh from concentrated 20× stock solutions. Be sure to check any thawed tubes for precipitate and vortex to re-dissolve as needed. Place on ice to bring to 0°C.
   b. As above, wash *Hydra* with decanted 0°C ddH_2O using a glass Pasteur pipette to agitate the *Hydra*, create a whirlpool to collect *Hydra* in the center of the dish, then remove media.

⚠ CRITICAL: It is important to work rapidly during this step to minimize damage to *Hydra*. Prolonged exposure to low osmolarity media devoid of calcium leads to the animal's eventual disintegration. Ideally, by the final wash *Hydra* will be on the verge of disintegration, and perhaps some have lost a tentacle or two, but should otherwise look the same as at the start of the wash.

⚠ CRITICAL: Terminate the experiment if large chunks or wisps of *Hydra* tissue appear in the media, or if the media clouds, a sign premature disintegration and osmotic shock-induced damage to cells. Troubleshooting 3.

Note: A helpful sub-experiment here includes leaving a hydranth or two behind in ddH_2O during the next step to see how they respond to extended incubation and manipulation in the lab's source of ultrapure water. Note that *Hydra* will appear contracted with a slightly 'rough/fuzzy' outline as they begin to disintegrate, at which point it is too late to use them for reaggregation. Anything beyond 'early depression' in Johnson and Gabel's *Hydra* Health Index should not be used in experiments (Bossert and Galliot, 2012; Johnson and Gabel, 1982).

4. Suspend *Hydra* in dissociation media and incubate at 4°C (Methods Video S2).
   a. After the last ddH_2O replacement, collect *Hydra* in the center of the dish by gently whirlpooling them with Pasteur pipette and transfer to a pre-chilled 1.5 mL microcentrifuge tube, minimizing ddH_2O carryover.
   b. Let *Hydra* sediment to the bottom of the tube and remove as much media as possible.
   c. Using a Pasteur pipette, bring the volume of the tube to 1.5 mL with ice-cold dissociation media to dilute the remaining H_2O and again allow *Hydra* to settle before removing as much media as possible.
   d. Again bring the volume of the tube to approximately 1.5 mL with ice-cold dissociation media. Aspirate the *Hydra* approximately 10 times into and from the pipette, ideally just to the point where fragments of cuticle or detached tentacles appear (Refer to the beginning of Methods Video S3 for mild trituration technique).

Note: Detached tentacles are not a cause for concern – some protocols even call for the removal of heads/tentacles and basal disks from *Hydra* before dissociation, although we have found this unnecessary (Soriano et al., 2009; Cochet-Escartin et al., 2017). Furthermore, altering the composition of aggregates with biases toward particular body structures (e.g., aggregates composed solely of anterior or posterior halves) produces aggregates with strikingly different phenotypes (Gierer et al., 1972).

   e. As before, allow the *Hydra* to settle at the bottom of the tube and remove as much media and debris as possible without disturbing the *Hydra*. Resuspend in roughly 1.3–1.5 mL fresh, ice-cold dissociation media, leaving empty space at the top of the tube to create a bubble to allow agitation of *Hydra* with inversion of the tube.

f. Resuspend and redistribute the *Hydra* by inverting the closed tube several times. Place the tube on its side in a 4°C refrigerator to avoid collection of *Hydra* at the bottom of the tube.
g. As before, replace dissociation media every half hour, a total of three times before completion of the 2-h 4°C incubation. Before every replacement, aspirate the *Hydra* gently, depending on the amount of tissue debris, around 10 times before allowing the animals to sediment, removing as much media as possible, adding fresh media to 1.3–1.5 mL and redistributing the *Hydra* by inverting the closed tube, as before.

Note: During this gentle trituration/aspiration, media should remain mostly clear aside from a slight bit of debris from either detached cuticle or tentacles. Individual cells that would cloud the media should not be liberated at this time.

Pause point: The four thirty-minute breaks between media exchanges allow completion of other tasks, although the last break can be devoted to preparing materials for the next step, initially described in 'Before You Begin' step 4c.

⚠ CRITICAL: Work steadily and rapidly during the ice-cold ddH_2O washes (Methods Video S2). Prolonged exposure here can lead to disintegration of *Hydra* into the highly hypoosmotic solution. A moderate amount of rigor during the washes and transfer is thought to loosen tissue to allow relatively isosmotic dissociation media penetrate during the incubation. Troubleshooting 3.

Dissociate *Hydra*

Timing: ~ 1 h

Pre-treated *Hydra* are partially dissociated, media is exchanged, and then *Hydra* are fully dissociated by mechanical trituration. Cell suspension is collected, and cells are counted and then seeded into tubes for sedimentation and reaggregation. Methods Videos S3 and S4 accompany this part of the protocol.

5. Exchange the dissociation media a final time after a pre-dissociation (Methods Video S3).
   a. Collect the *Hydra*-containing tube from the 4°C incubation and place on ice.
   b. Compressing the bulb, angle the tip of an unpolished, fresh Pasteur pipette toward the bottom of the microcentrifuge tube containing the *Hydra*, resting the side of the pipette against the lip of the tube opening, and placing the bore of the pipette as close to the bottom as possible without creating a seal.

Note: It is thought that the sharp edge of a fresh pipette provides a measure of standardization and helps shear the *Hydra* during dissociation, which may not progress as planned if the tip is flame-polished or is gummed with debris from previous use. Substitution of other trituration instruments (e.g., plastic transfer pipettes, P1000 tips) is theoretically possible but will require further optimization. Troubleshooting 1.

   c. Draw approximately 90% of the contents of the tube (media, animals) into the pipette, and expel the contents, holding the same angle of the pipette tip, without emptying the entire volume to avoid the production of bubbles.

Note: More volume can be drawn into the Pasteur pipette by stretching a compressed bulb before insertion into the incubation tube. Ideally one is able to draw approximately 90% of the contents into the pipette, to maximize the trituration of tissue.

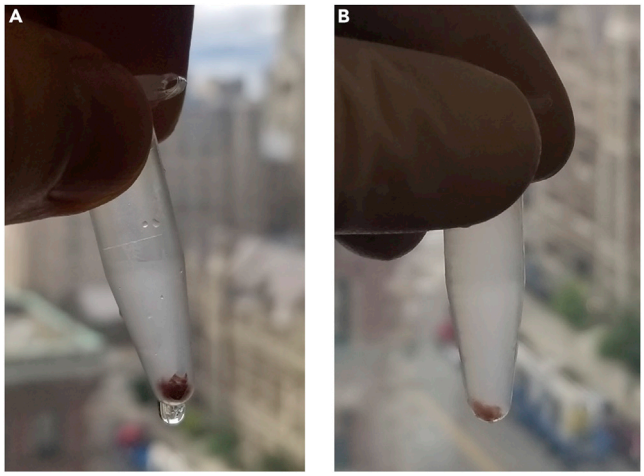

Figure 4. *Hydra* before and after dissociation

(A) ~70 pre-dissociation *Hydra* sedimented at the bottom of a 1.5 mL microcentrifuge tube prior to the final pre-dissociation media exchange. Note the volume of cells prior to dissociation and the slight cloudiness of the media from ~20 pre-dissociation pipette plunges to bring the animals to the verge of dissociation.

(B) Sedimented *Hydra* tissue fragments post-dissociation. Note again the volume of the fragments. Ideal trituration (in this case, 60 plunges of the Pasteur pipette) aims to dissociate between 2/3 and 3/4 of the initial volume of *Hydra*. Note also the increased cloudiness of the media compared to A and Figure 6A.

d. Repeat this process 10–20×, maintaining the angle of the pipette and avoiding the generation of bubbles with the pipette, until the media starts to cloud, indicating the start of the dissociation of the animal. A rate of approximately one complete plunging cycle per second is a good benchmark.
e. Allow the animals to sediment and replace the now slightly cloudy dissociation media with fresh dissociation media before the actual dissociation, noting the volume of intact animal tissue at the bottom of the microcentrifuge tube (Figure 4A).

Note: The goal for the next step is to optimally capture the most cells with the fewest number of plunges, beginning the actual cell collection as the rate of cell dissociation begins to rapidly increase. This sacrifices some dissociated cells from this step that would otherwise be over-triturated and potentially not viable in aggregates.

6. Dissociate the *Hydra* into individual cells (Methods Video S3).
   a. As before, expel air from the plunger and insert the pipette into the tube in the position described above. Draw approximately 90% of the contents into the pipette and expel, as before, avoiding bubbles.
   b. Repeat the process 50–100×, until the media is nearly opaque.

Note: A bright background (e.g., performing the dissociation next to an open window) helps monitor progress – the cloudiness of the media is more easily observed in the microcentrifuge tube and pipette.

⚠ CRITICAL: It is important to be firm with the pipetting so the tissue actually dissociates. A fair amount of tissue damage is expected, and in most cases aggregates will recover, even with massive cell death during the process. Dying cells are eventually sorted and excluded from the regenerating animal. Conversely, overly-aggressive pipetting can lead to levels of cell death that cannot be overcome. Again, a rate of approximately one complete plunging cycle per second is a good benchmark. Troubleshooting 3.

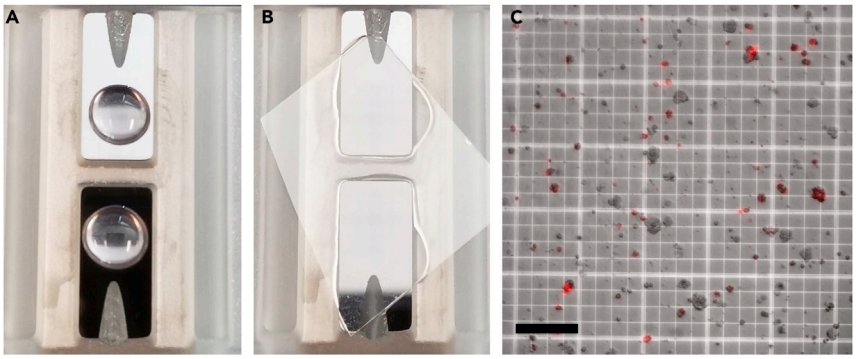

Figure 5. Counting of dissociated *Hydra* cells

(A) Cell suspension is placed directly onto the counting chamber, as opposed to using the capillary action of the divots on the top and bottom of the chamber, as might be done with standard laboratory cell lines. This ensures any larger tissue fragments that might not be pulled into the counting chamber via capillary action don't lead to underestimating the concentration of the cell suspension.

(B) The coverslip of the counting chamber is placed directly onto the cell suspension gently, as opposed to positioning it before loading cells via capillary action.

(C) Approximately ~250 cells are present in the 5 × 5 0.1 μL counting grid, leading to a final concentration of 2.5 million cells/mL. Note the diversity of cell types and sizes and that interstitial cells are much smaller and much more transparent than epitheliomuscular cells, the darkest of which are the endodermal cells (highlighted here in red, as the dissociation was performed on a *Hydra* line expressing RCaMP in the endodermal cell linage. Scale bar 200 μm.

7. Sediment tissue fragments on ice, collect the cell supernatant (Methods Video S4).
   a. Place the tube containing dissociated *Hydra* on ice for 1 min.

   ⚠ CRITICAL: Check the final volume of the undissociated tissue fragments. A good benchmark is 1/4 to 1/3 of the volume of tissue compared to the volume immediately prior to dissociation (Figure 4B). Volumes larger than this may indicate incomplete dissociation and potential unrepresentative ratios of epithelial cells. Volumes less than this may indicate over-trituration with levels of cell death that will not allow regeneration.

   b. Rinse the pipette tip to remove any residual adherent tissue fragments and carefully collect the cell-containing supernatant, taking care to not disturb the tissue sediment.

   Note: Longer sedimentation times will allow increasingly smaller tissue fragments to fall out of suspension, at the expense of individual dissociated cells that sediment during the time period as well.

8. Count the cells using the hemocytometer.
   a. Gently resuspend the cell suspension in the 1.5 mL tube with the Pasteur pipette to homogenize and add one drop to each side of the hemocytometer.

   Note: Drops should be placed directly on the counting grid as opposed to using the capillary action of the divots of the hemocytometer as larger chunks of cells, if present, may not be pulled to the measuring grid (Figure 5A). Troubleshooting 5.

   b. Count the cells to determine the concentration. Note any clumps of cells, if present.
      i. A representative result will have 5%–10% of the total number of cells in small clumps of 5–10 cells, sometimes larger. Motile and adhesive cells begin reaggregating immediately after dissociation.

Note: Some protocols call for the 40 μm filtration of cells. This diameter is already larger than many of the small clumps of cells, given that *Hydra* cells are relatively small. It has been observed that the distribution of clumps to individual dissociated cells doesn't change after filtration, unless there are excessively large clumps, in which case the dissociation is incomplete (Figure 5C).

ii. A representative result will give 1.5–5 million cells/mL depending on the initial number of *Hydra* used in the experiment and the total fraction of tissue dissociated.

9. Allocate cells for aggregates of desired size to pre-chilled 0.5 mL microcentrifuge tubes at a final volume of 200 μL and sediment on ice 30 min.
   a. Determine the volume to add to achieve aggregates of the sizes required of the experiment.
      i. Aggregates of 80–100 thousand cells fit comfortably in the field of most 10× microscope objectives. Troubleshooting 5.

Note: Aggregates smaller than 50 thousand cells have a harder time surviving (Gierer et al., 1972). In theory aggregates can be made of any number of cells greater than this. If there is massive cell death, viable cells can find each other in larger masses of dying cells to form multiple distinct aggregates within the larger cell mass. Troubleshooting 3.

   b. Determine the volume of ice-cold dissociation media to add to the volume of cell suspension to yield a 200 μL final volume, and add this to pre-chilled 0.5 mL microcentrifuge tubes before adding the cell suspension.
   c. Add the cell suspension gently to the bottom of the tube, and allow cells to sediment on ice for 30 min. Troubleshooting 4.

Pause point: 30 min.

Optional: If the dissociation did not yield adequate numbers of cells, the remaining tissue fragments can be resuspended in dissociation media and incubated at 4°C for a longer period of time, repeating steps 5 through 9.

Cellular aggregate creation and maintenance

Timing: 8–72 h

10. After the 30 min sedimentation of the cell suspension on ice, centrifuge the cell suspension slowly for 4 min at 300 g.
    a. Transfer the tubes to the centrifuge slowly with care so as not to disturb the previous 30-min bout of sedimentation. Troubleshooting 4.
    b. Centrifuge without a lid, if possible, to avoid disturbing the cell pellets with attachment and detachment of the lid. At such a low speed as 300 g, this should not damage the centrifuge.

Note: A non-refrigerated centrifuge can be used here with no adverse effects on preparation quality.

Alternatives: A wide variety of centrifugation speeds and even alternative approaches exist for the generation of aggregates from cell suspension. At the higher end is 1,000 × *g* for 5 min (Cochet-Escartin et al., 2017). At the lower end is 10 × *g* for 10 min (Soriano et al., 2009). Others (Technau and Holstein, 1992; Hobmayer et al., 2001) have used a rotary shaker on cell suspension in a 24 well plate to create cellular aggregates.

Pause point: 4 min.

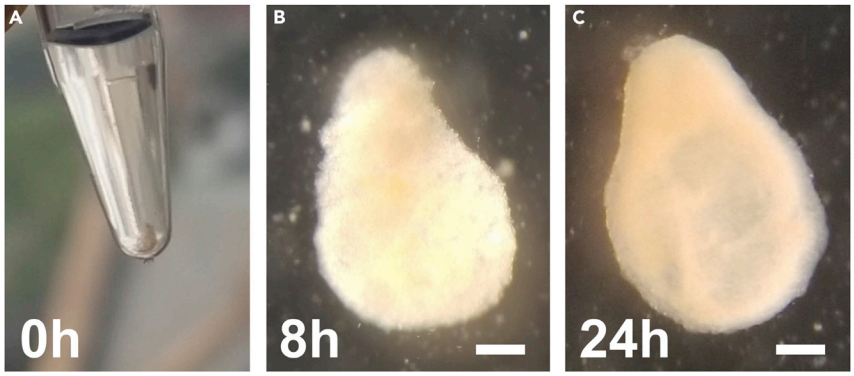

Figure 6. Representative *Hydra* aggregates

(A) Pelleted cell suspension of 100 thousand cells in 200 μL dissociation media, following a 30-min incubation of the cell suspension on ice and 4-min centrifugation at 300 g in a fixed-angle rotor. Shorter incubations of the cell suspension on ice before centrifugation lead to longer streaks toward the top of the pellet and increasingly oblong aggregates.

(B) An aggregate 8 h post-dissociation.

(C) An aggregate 24 h post-dissociation. Note the clearer segregation between the ectoderm (translucent halo) and the endoderm, as well as the hollowing center of the aggregate at 24 h. Some shedding of unintegrated cells is observed in both cases. Scale bar 200 μm.

11. After centrifugation, gently place the 0.5 mL microcentrifuge tubes in their rack (the repurposed P1000 tip box), cover, and place at 4°C for 2–6 h to allow the early stages of reaggregation to progress undisturbed.
    a. Inspect the microcentrifuge tubes for cell pellets to ensure sedimentation and aggregation (Figure 6A). Shorter post-allocation incubation periods of the cell suspension can lead to streaking of cells in a thin layer along the side of the tube, loss of cells from the aggregate, and oblong aggregates. Troubleshooting 4.
    b. If desired, add ice to the bottom of the empty 1 mL pipette tip box serving as a 0.5 mL tube holder to slow the temperature transition to 4°C and eventually to 18°C.

Note: Some protocols call for the collection of aggregates immediately after centrifugation. We have found this difficult, and the process can lead to major cell loss and variable aggregate sizes. Incubation at 4°C gives the aggregate time to develop and detach from the tube, to facilitate the eventual transfer to 24-well plates.

Optional: The 4°C incubation can be substituted for an 18°C incubation with no severe effects on aggregate health (Lovas and Yuste, 2021). However, it has been observed that aggregates are more robust to experimental manipulations and appear to regenerate faster with a slower transition from 4°C back to standard incubation at 18°C. Equilibrating the dissociation medium-containing 24-well plates to 18°C instead of 0°C is performed in the next step if the extended 4°C incubation is omitted.

12. After 4°C incubation, transfer aggregates to 24-well plates for incubation at 18°C.
    a. Pre-chill the 24-well plate on ice and add 600 μL ice-cold dissociation medium.
        i. This minimal volume minimizes media waste and will completely cover the bottom of the 24-well plate.
    b. Gently bounce the bottoms of the tubes in the 1 mL tip holder/tube rack on a flat surface a few times to facilitate detachment of aggregates from the tubes. Take care not to lose tubes from the rack.
    c. Transfer the 200 μL contents of the 0.5 mL microcentrifuge tube to the 600 μL dissociation medium in a well of the 24-well plate. Inspect the Pasteur pipette to ensure the aggregate is pulled from the bottom before the tube is empty.

d. Place the 24-well plate in an 18°C incubator.

Note: Aggregates usually detach readily with gentle aspiration of the Pasteur pipette, but can be gently nudged with the pipette tip if they don't immediately detach. Take care to ensure aggregates aren't exposed to air or surface tension during the process. Troubleshooting 4.

13. Gradually dilute dissociation media back to normal *Hydra* media supplemented with Penicillin/Streptomycin. Troubleshooting 2.
    a. A typical schedule is a 50% dilution at 6 h, 22 h, 46 h, and 70 h post-sedimentation.
    b. To avoid disturbing the fragile new aggregates, the first 50% dilution at 8 h should involve gently adding another 800 μL *Hydra* media to the 800 μL dissociation media in the well. Subsequent dilutions should include removal of 800 μL media before replacement.

Alternatives: The dilution schedule for bringing the aggregates back to low-osmolarity *Hydra* media varies widely from protocol to protocol. At one extreme, after centrifugation aggregates are left to stabilize in a 50% dilution before transfer to 100% *Hydra* medium at 2 h (Soriano et al., 2009). The protocol presented here is near the other end of the spectrum. We have observed that intact *Hydra* are content for extended periods of time in 'Recovery Media' post-electroporation – 20% dissociation media in *Hydra* media (Bosch et al., 2002) – as evidenced by their exceptionally long tentacles. A more frequent dilution schedule (e.g., every 12 h after the first exchange) has no adverse effects on aggregate health. If massive cell shedding is present a more frequent schedule may be desired to allow the removal of debris.

EXPECTED OUTCOMES

Hydra was first observed to reaggregate and regenerate after dissociation into individual cells in the early 1970s (Noda, 1971; Gierer et al., 1972). Starting with healthy, clean *Hydra* and subjecting them to an appropriate level of dissociation, survival of all aggregates formed during an experiment is expected. By 8 h, aggregates should have rounded into amorphous spheroids, and early segregation of the endoderm (translucent border) and endoderm (pink center of the aggregate) should be present (Figure 6B). By 24 h, the aggregates should be largely hollow in the middle, with much clearer, near-complete segregation of the endoderm and ectoderm (Figure 6C). By 48 h, a number of cells may be present in the gastric cavity, likely dependent on the amount of cell damage incurred during trituration. Aggregates may be less spherical between 48 h and 72 h, as this is when the symmetry-breaking event that establishes the body axes of the regenerating animal occurs (Methods Video S5). In most cases at 72 h aggregates will display coordinated contractions of the entire animal (Lovas and Yuste, 2021).

At all time points, depending on the amount of cell damage incurred during the process, some degree of cell shedding is expected, either to the area surrounding the aggregate or to the interior of the gastric cavity (Figure 6). These loose cells can be collected during and after the second media exchange at 24 h. From ~36–60 h, if *Hydra* are compressed during imaging, if cells are present in the gut they may extrude if the aggregate bursts. A normal 'swelling and popping' of aggregates is expected – thought to be involved in osmoregulation and body axis reestablishment (Soriano et al., 2009).

After the last media exchange at 72 h, the regenerating *Hydra* can be cultured normally. Regenerating hydranths should have developed tentacles and a mouth capable of feeding by 1 week at the most, sometimes earlier, depending on the strain of animal used (Methods Video S5). Depending on the number of cells that form the aggregate, 1 or more *Hydra* will eventually emerge from the aggregate. Small aggregates of 100k cells usually give one small animal, sometimes two very small animals. Aggregates larger than this are increasingly likely to yield more body axes and eventually more individual animals. By 2 weeks, larger aggregates will have completely segregated into individuals largely indistinguishable from the starting population.

LIMITATIONS

The largest source of variability in the results of the protocol stems from the individual manual calibration required to triturate the *Hydra* tissue during the primary dissociation to appropriate levels. Too soft, and the *Hydra* won't dissociate. Too firm, and an increasing amount of cell damage poses risks to aggregate health. Future efforts to scale the process might involve the engineering of a more 'hands-free' apparatus or technique to standardize the procedure.

Beyond this, throughout the process of *Hydra* culture there are several factors that may inject variability into experiment results. As one example, *Hydra* are generally fed *ad libitum* after addition of *Artemia* nauplii to their culture dish, and a variety of factors thereafter can influence how much individual hydranths will eat, leading to variability in the nutritional status of animals. Moreover, culture conditions beyond the experimenter's control can influence the health of animals – we have observed that *Hydra* are generally healthier in Woods Hole, MA, compared to New York, NY. We speculate this difference is due to trace minerals in water sources or the climate of each region.

Taken together, these factors create a situation where the experiment will occasionally fail for no discernable reason. It is here we encourage persistence. The novice experimenter might begin with a higher degree of 'chunks' of tissue in their aggregates, by using 100–150 animals as opposed to the recommended 50–70, not dissociating the preparation quite as thoroughly and allowing tissue fragments post-dissociation to sediment for a shorter amount of time.

TROUBLESHOOTING

Problem 1

Hydra are not dissociating, even with extended trituration.

Potential solutions

Check the feeding schedule of the parent colony. Be sure the *Hydra* have been fed no more than 72 h before dissociation (step 1a). Empirically, the dissociation doesn't progress as readily if animals have been starved longer than 72 h. The dissociation then requires more vigorous trituration to break apart the 'stickier' animals, and larger chunks and fragments of *Hydra* are present in the final cell suspension.

Confirm pH and composition of the dissociation medium (step 3a). *Hydra* are freshwater Cnidarians and spontaneously disintegrate in saline. Standard *Hydra* media has an osmolarity of ~5mOsm. Dissociation media is ~65mOsm. If salts precipitate from the dissociation media or it is prepared incorrectly, dissociation may not progress.

Check the number and size of *Hydra* used for dissociation (step 1). The protocol presented here is optimized for roughly 50-70 'medium' *Hydra*, or a representative distribution of small through large *Hydra*. If too few *Hydra* pass through the bore of the pipette tip, dissociation becomes more difficult. Conversely, too many *Hydra* will lead to incomplete dissociation and larger chunks of tissue.

Confirm Pasteur pipette quality. Be sure to use a fresh, unpolished 15 cm pipette (step 5b). We have observed that the use of a flame-polished tip on a longer Pasteur pipette will not dissociate *Hydra*, while use of a fresh, unpolished pipette on the same preparation will lead to dissociation (Methods Video S3).

Problem 2

Aggregates fall apart between 24- and 48 h. Microbial contamination is suspected.

Potential solution

Confirm *Hydra* colony health and begin with a clean preparation (step 2, Figure 1). Any infection of the colony or excessive microbial growth likely won't be eliminated by the washing steps or

supplementing dissociation medium with Penicillin/Streptomycin, carrying the infection into the aggregates. Microbial contamination can be confirmed by monitoring failed aggregates with high magnification microscopy after their disintegration to observe any microbial growth.

Infections that don't have a noticeable effect on the health of intact animals may appear suddenly when working with more fragile preparations – aggregates or new hatchlings (Franzenburg et al., 2013). Additionally, extra care should be taken with antibiotic treated animals or aggregates, as they are at increased susceptibility to fungal infections (Fraune et al., 2015).

Problem 3

Aggregates fall apart between 24- and 48 h. Microbial contamination is not suspected.

Potential solution

Confirm the pH and composition of dissociation media (step 3) and number and size of *Hydra* used in the experiment (step 1). Check the intensity of mechanical trituration (step 6; Methods Video S3). The process of dissociation is incredibly traumatic, and some level of cell death is expected and as reaggregatoin progresses will be observed through the shedding of cells and the collection of unintegrated cells in the newly formed gut of the animal. If too much cell death is present, however, reaggregation won't progress and the aggregates will eventually fall apart. An ideal dissociation will progress to 2/3–3/4 dissociated tissue with 50–100 plunges of the Pasteur pipette (Figure 4, Methods Videos S3 and S4). If more plunges are required to dissociate the tissue, this risks further damaging already liberated cells as they are excessively agitated.

Another potential source of failure comes from osmotic shock during the initial ice-cold di-H_2O washes before incubation in dissociation medium at 4°C (step 3). If *Hydra* are left too long in the water, or if the water isn't ice cold, cells will begin to lyse and *Hydra* will begin to disintegrate. Working rapidly without pause during the washes is essential here, as well as ensuring the water is ice-cold. We recommend placing a glass vessel on ice well in advance of animal collection and the initial washes with room-temperature *Hydra* media to accomplish this.

As a starting point for the novice experimenter, larger aggregates (200–300k cells) are generally more robust. Additionally, incorporating more animals into the dissociation (100–150 vs. the recommended 50–70) will give more cells with fewer plunges, and will give larger 'chunks' of incompletely dissociated tissue or larger clumps of cells that will regenerate more readily, if appropriate for the experiment.

Problem 4

Aggregates are oblong or oddly shaped after centrifuging the cell suspension.

Potential solution

After allocating the cell suspension to 0.5 mL tubes for individual aggregates, if the cells don't sediment enough before centrifuging, an increasing amount of cell smearing on the side of the tube will be observed, particularly when using a fixed-angle rotor (step 10). Ensure the sedimentation takes place on a vibration free table. The sedimentation can also be extended to 45 min, potentially longer, with no adverse effect on aggregate quality.

Additionally, even with enough sedimentation, aggregates will often resemble 'flakes' or 'pancakes' depending on their size. While this is not a concern and aggregates will rearrange themselves into hollow spheres quickly – usually within the first eight hours – it can make the aggregates difficult to handle and complicates transfer from the 0.5 mL tube to their final 1/24 well if they are larger than the pipette bore. If the aggregates have had enough time to incubate at 4°C before plating (~6 h) they will be more resilient after passing through a bore smaller than their diameter. Alternatively, with enough time to incubate aggregates can be gently detached and resuspended with the edge or

stream of a smaller pipette tip (e.g., P1000), and transferred with the larger non-tapered end of the Pasteur pipette (step 11).

If it does not directly interfere with experiments, aggregates can also be resected after they are initially formed. Indeed, some instantiations of the protocol prepare all aggregates this way, cutting individual aggregate-sized pieces from a cell-mass extruded from a thin centrifugation tube (Gierer et al., 1972; Seybold et al., 2016). Caution should be taken during this process, as shards of autofluorescent plastic liberated from the dish by a scalpel blade can become lodged in the aggregate during resection.

Problem 5

Aggregates are not the correct size for experiments.

Potential solution

Controlling the amount of damage to cells during the dissociation is difficult – a variable number of dead and dying cells are sorted and shed from the aggregate as the morphallaxis progresses. In addition, the presence of any 'clumps' of cells can complicate counting, if the true number of cells is over- or underestimated (step 9). This leads to a situation in which aggregates are sometimes not the correct size for experiments (e.g., slightly too large to fit completely in a microscope field of view).

While there will be some variability from aggregate to aggregate, one safe way to approach this problem is to generate extra aggregates of different sizes, varying the size of each by 20% (e.g., making 80k, 100k, and 120k-cell aggregates, if a 100k-cell aggregate is desired).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Rafael Yuste (rmy5@columbia.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze any new data or code. Please refer to Lovas and Yuste (2021) for data and code related to the study of neural circuit synchronization during reaggregation.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101504.

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AUTHOR CONTRIBUTIONS

J.R.L. and R.Y. conceptualized the work. J.R.L. conducted experiments and parameter optimization. J.R.L. wrote the initial manuscript. J.R.L. and R.Y. edited the manuscript. R.Y. directed the project and secured funding.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

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