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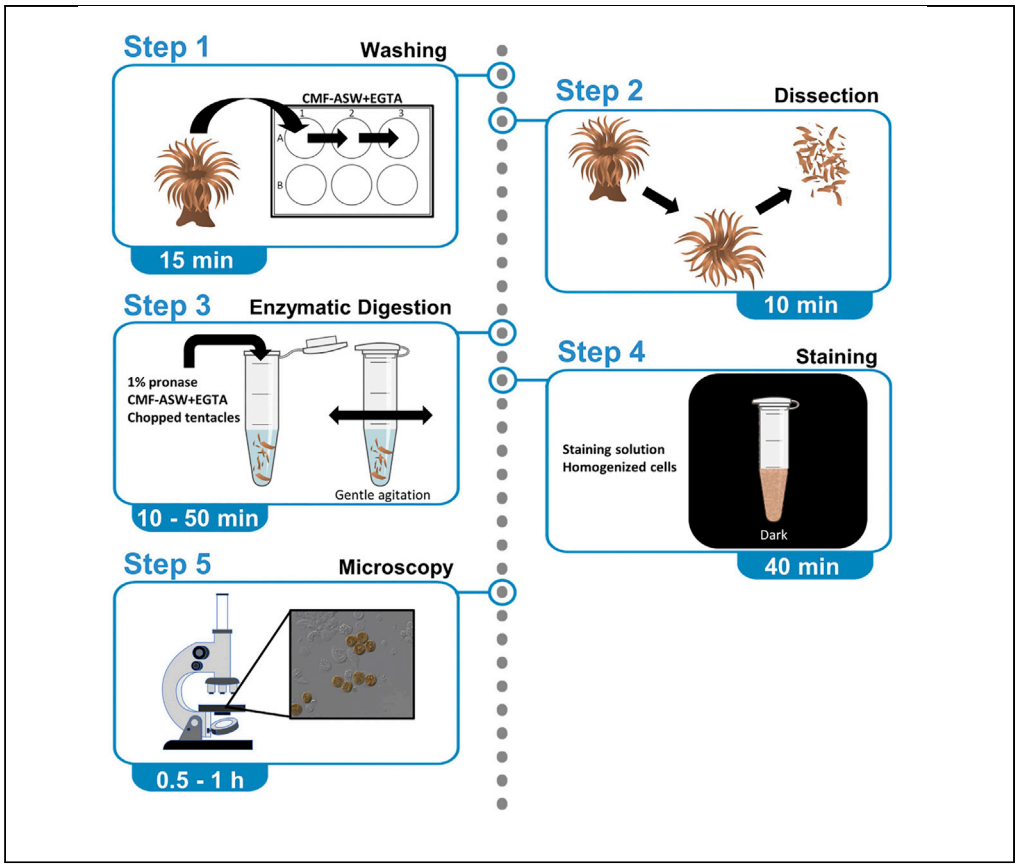
Single-cell dissociation of the model cnidarian sea anemone *Exaiptasia diaphana*



The sea anemone *Exaiptasia diaphana* (Aiptasia) is a versatile model in studying cellular mechanisms that govern cnidarian-Symbiodiniaceae symbiosis, the foundation of coral reef ecosystems. Here, we provide a protocol to efficiently dissociate adult Aiptasia tissue into a single-cell suspension using enzymatic digestion. We detail steps including washing animals, dissociating tissue with pronase digestion, and evaluating dissociated single cells using fluorescence imaging. This procedure can be applied to other cnidarians, including coral polyps.

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

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Highlights

Procedure for an efficient dissociation of sea anemone and coral tissue

A linear model depicting the correlation between dissociation time and tentacle mass

Isolation of a single-cell suspension containing intact Aiptasia host cells

Protocol for fluorescent staining and microscopy of dissociated single cells

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Single-cell dissociation of the model cnidarian sea anemone *Exaiptasia diaphana*

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SUMMARY

The sea anemone *Exaiptasia diaphana* (Aiptasia) is a versatile model in studying cellular mechanisms that govern cnidarian-Symbiodiniaceae symbiosis, the foundation of coral reef ecosystems. Here, we provide a protocol to efficiently dissociate adult Aiptasia tissue into a single-cell suspension using enzymatic digestion. We detail steps including washing animals, dissociating tissue with pronase digestion, and evaluating dissociated single cells using fluorescence imaging. This procedure can be applied to other cnidarians, including coral polyps.

For complete details on the use and execution of this protocol, please refer to Jinkerson et al. (2022).[1]

BEFORE YOU BEGIN

Timing: 0.5–2 h

1. Prepare all reagents and buffers:
   a. Calcium- and magnesium-free artificial seawater (CMF-ASW) with EGTA.
   b. 1% pronase solution.
   c. If desired, staining cocktail.
2. Review and become familiar with each step of the protocol, as they should be completed quickly to avoid over digestion of the Aiptasia tissue.

Note: This protocol is optimized for adult Aiptasia but can also be used with coral polyps.

Note: We have demonstrated the protocol using one animal, but the protocol can be scaled up to dissociate multiple animals at once.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
EGTA, molecular biology grade (ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid)	MilliporeSigma	Cat# 324626
HEPES	Sigma-Aldrich	Cat# H4034

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Tris HCl, 1 M Solutions, pH 8.0	Fisher	Cat# BP1758
Sodium chloride	Sigma-Aldrich	Cat# S3014
Potassium chloride	Fisher	Cat# 02151944
Sodium sulfate	Fisher	Cat# S415
Sodium bicarbonate	Fisher	Cat# S233
Pronase from *Streptomyces griseus* (~7.0 units/mg protein at 40°C with casein as the substrate, pH 7.5)	MilliporeSigma	Cat# 10165921001
Hoechst 33342 fluorescent nucleic acid stain	Thermo Fisher	Cat# H3570
Experimental models: Organisms/strains		
Clonal axenic *Breviolum minutum* (Clade B) strain SSB01	The Aiptasia Symbiosis Resource	SSB01
Exaiptasia diaphana strain CC7, but the provided protocol will also work with H2 or other anemone strains	The Aiptasia Symbiosis Resource	CC7
Software and algorithms		
ImageJ	https://imagej.nih.gov/ij/	https://imagej.nih.gov/ij/download.html
R Software (version 4.1.1)	https://www.R-project.org/	https://cloud.r-project.org/
Other		
Micropipettes	VWR	N/A
Single-edge razor blade	N/A	N/A
Eppendorf centrifuge 5425	Eppendorf	Cat# 5405000107
6-well plates	Greiner	N/A
Leica S9E (dissection microscope)	Leica Microsystems	N/A
Orion Star™ A214 pH/ISE Benchtop Meter	Thermo Fisher	Cat# STARA214
Orbital shaker or rocker	N/A	N/A
Petri dish	VWR	Cat# 89038
Tweezers	N/A	N/A
22 mm × 22 mm coverslips	VWR	Cat# 48366
Microscope slides	VWR	Cat# 16005
Zeiss Axio Observer inverted microscope	Carl Zeiss	N/A
Transfer pipets, disposable polyethylene	Fisher	Cat# 13-711-7M
Clear nail polish	N/A	N/A

MATERIALS AND EQUIPMENT

Calcium- and Magnesium Free Artificial Seawater with EGTA (CMF-ASW+EGTA)[2]		
Reagent	Final concentration	Amount
Sodium chloride	450 mM	13.1 g
Potassium chloride	9 mM	0.34 g
Sodium sulfate	30 mM	2.31 g
Sodium Bicarbonate	2.5 mM	0.105 g
Tris HCl, 1 M, pH 8.0	10 mM	5 mL
EGTA, 0.2 M, pH 8.0	2.5 mM	6.25 mL
HEPES, 1 M, pH 6.9	25 mM	12.5 mL
Milli-Q® ultrapure (Type 1, 18.2 MΩ·cm) water	N/A	Up to 500 mL
Total	**N/A**	**500 mL**
Prepare ahead of time, adjust pH to 8.0, store at 4°C for up to six months, and bring to 25°C for protocol steps.		

1% Pronase in CMF-ASW+EGTA		
Reagent	Final concentration	Amount
Pronase	1% (w/v)	40 mg
CMF-ASW+EGTA	N/A	4 mL
Total	**N/A**	**4 mL**
Prepare ahead of time, divide into 400 µL aliquots, and store at −20°C for up to one year.		

Staining cocktail		
Reagent	Final concentration	Amount
Hoechst 33342, 1 mg/mL	12 µg/mL	6 µL
CMF-ASW+EGTA	N/A	494 µL
Total	**N/A**	**500 µL**
Prepare ahead of time, and store at −20°C.		

⚠ CRITICAL: Pronase is an irritant to skin, eyes, and the respiratory system. Wear gloves and use in a well-ventilated area such as a fume hood.

STEP-BY-STEP METHOD DETAILS

Washing of whole Aiptasia

Timing: 15 min

Washing the animals ensures that external particles (i.e., food or expelled algae) and the calcium/magnesium containing seawater are removed from the anemone. As calcium and magnesium promote cell-cell adhesion, reducing these ions ensures efficient dissociation of single-cells.

1. Select one adult Aiptasia approximately 0.5–1.5 cm in total diameter, including tentacles (Figure 1).
   a. Fill three wells of a 6-well plate with CMF-ASW+EGTA.
   b. Using a transfer pipette, move the selected animal to the first well of the prepared 6-well plate and wash by gently pipetting the animal in and out of the pipette. The animal may produce mucus at this time. Troubleshooting 1.

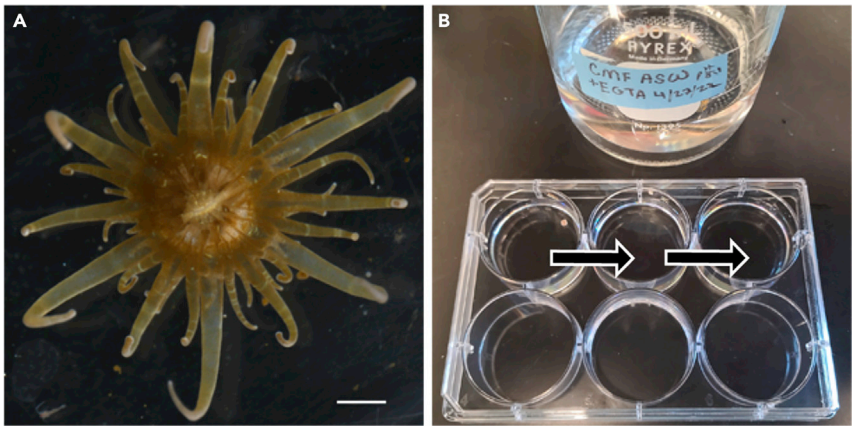

Figure 1. Washing Aiptasia in CMF-ASW+EGTA

(A) A representative microscopic image of adult CC7 Aiptasia symbiotic with *Breviolum minutum* (SSB01). Scale bar, 1 mm.
(B) The animal is serially transferred across the first three wells of a 6-well plate and allowed to incubate in the final well.

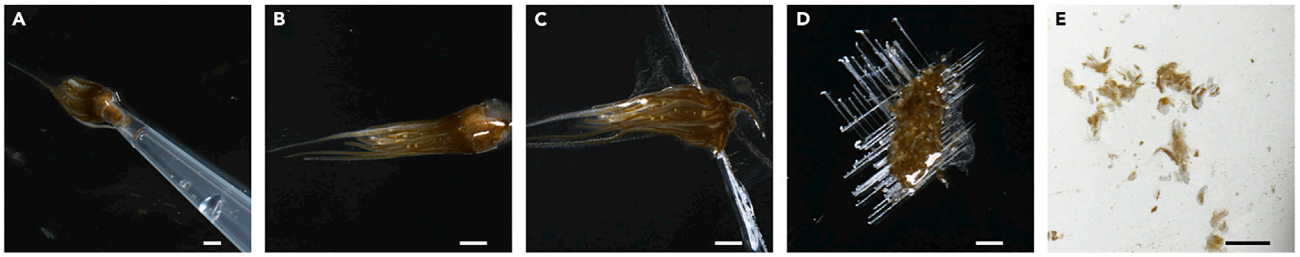

Figure 2. Dissection of Aiptasia tentacles

The tentacles are removed from the animal and chopped into fine pieces to increase surface area for enzymatic digestion. Scale bars, 1 mm.

(A) The animal is transferred to a petri dish with minimal liquid.

(B) The tentacles are oriented in one direction, and the animal is cut where the tentacles meet the body.

(C) The body is removed and discarded.

(D) The tentacles are chopped vertically and horizontally.

(E) Representative view of results after tentacles are chopped and resuspended in CMF-ASW+EGTA.

c. Repeat the washing process two additional times by transferring the anemone to the second and third wells of the 6-well plate. When transferring the animal between wells, take as little of the washing media as possible for each transfer.

Note: When moving the animal between wells, do not draw the animal into the pipette, simply use the suction from the pipette to lift the animal out of the well and drop it into the next well for washing. This will minimize the transfer of washing media and debris between wells.

⚠ CRITICAL: If mucus or external particles remain on the animal at this stage, repeat steps 1a–1c until little to no mucus is observed on the animal. Mucus will interfere with the downstream steps of cutting and efficient centrifugation.

2. After the final wash, allow the animal to incubate in the last well for at least 10 min.

Pause point: The animal can remain in the CMF-ASW+EGTA for up to 3 h.

Tentacle dissection

Timing: 10 min

Mechanical separation of the Aiptasia tissue is necessary to increase the surface area of host tissue exposed to pronase in later stages of the protocol and enhances the number of host cells successfully dissociated.

3. Using suction from a clean transfer pipette, remove the Aiptasia anemone from the final washing well (taking minimal washing media) and place the animal on an empty, inverted petri dish (Figure 2).
   a. Under a dissection microscope, use the suction of a p200 pipette to orient the animal so that it is laying on its side.
   b. Dissect all of the tentacles from the body using a razor blade. Troubleshooting 2.
   c. Drag the body away from the tentacles and discard.
   d. Finely chop the tentacles horizontally and vertically with the razor blade.
   e. Use a small amount of CMF-ASW+EGTA (approximately 200 µL) to resuspend the chopped tentacle pieces and move them to a pre-weighed 1.5 mL tube.
4. Centrifuge the chopped tentacle pieces at 70 × *g* for 5 min in an Eppendorf centrifuge 5425 (Figure 3).

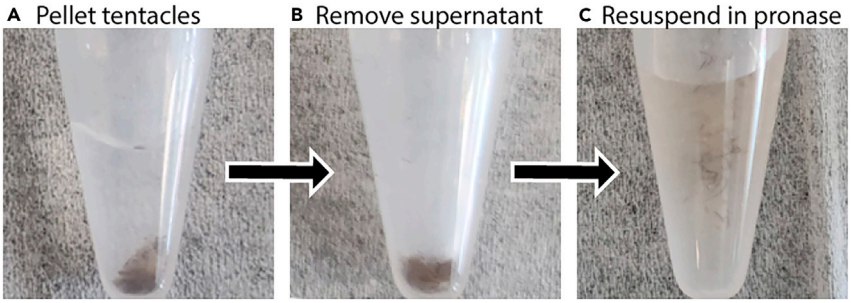

Figure 3. Preparation of dissected Aiptasia tentacles for enzymatic digestion
(A) The dissected tentacles form a pellet after centrifugation.
(B) The supernatant is removed from the pellet of tentacles.
(C) The tentacles are resuspended in pronase solution to begin enzymatic digestion.

5. Gently remove and discard the supernatant from the animal pellet (Figure 3). Troubleshooting 3.

Enzymatic digestion

Timing: 10–50 min

A low concentration of pronase will digest proteins and disrupt the cell-cell protein interactions to quickly dissociate the tissue into single cells.

6. Once the supernatant is removed, weigh the 1.5 mL tube containing the pelleted tentacles. Determine the tentacle mass in milligrams by subtracting the mass of the empty tube.
7. To the tissue pellet, add 400 µL of 0.5% pronase solution in CMF-ASW+EGTA.

Note: Prepare the 0.5% pronase solution fresh and use immediately by mixing 200 µL CMF-ASW+EGTA and 200 µL 1% pronase CMF-ASW+EGTA.

8. Mix slowly and gently by pipetting up and down with a p200 or p1000 pipette approximately eight times until the tentacle pieces are well mixed in the pronase solution (Figure 3).
9. Incubate the tentacle pieces on an orbital shaker at 25°C with gentle agitation for approximately 0.7 min per milligram of tentacle mass (Figure 4). Continue the incubation until the moment no visible tissue chunks remain in the solution.

Note: During the incubation, flick the tube or slowly pipette the solution up and down to mix the pronase solution and check the status of tissue dissociations (Figure 5). Troubleshooting 4.

⚠ CRITICAL: Avoid over-pipetting the mixture, as this will damage and rupture host cells. Avoid mixing more than twice during the incubation period.

10. After incubation, centrifuge the dissociated cells at 70 × *g* for 5 min. Gently remove the supernatant and discard. The supernatant should be clear, as shown in Figure 5.

Alternatives: To complete the dissociation and staining process simultaneously, the following steps should be followed:

a. Gently suspend the chopped tentacles in a final concentration of 0.25% w/v pronase and 20–30 µg/mL Hoechst 33342 in CMF-ASW+EGTA. Pipette the suspension up and down eight times.

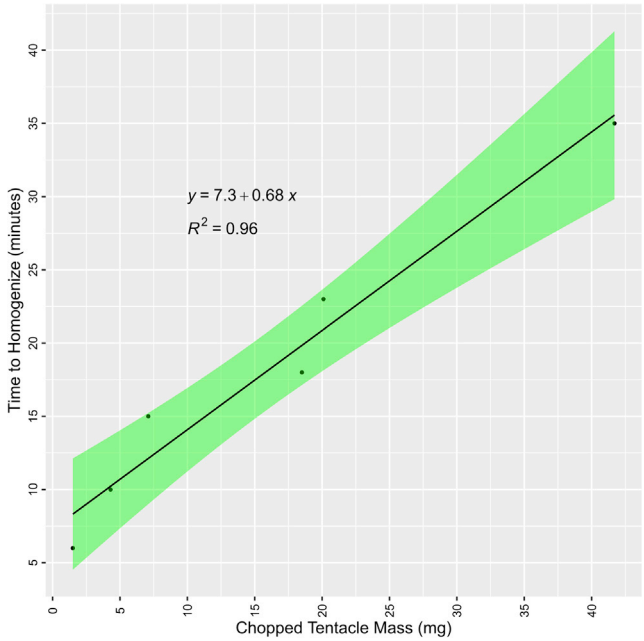

Figure 4. Approximate time for dissociation in relation to the tentacle mass
Linear model was generated using the ggplot2 package in R version 4.1.1.[3] Y axis represents the approximate time to reach a homogenized solution. X axis represents the mass of chopped tentacles used in the dissociation.

b. Incubate the tentacle pieces in dark, at 25°C, on an orbital shaker with gentle agitation for approximately 20–30 min, or until no visible tissue chunks remain.
c. Proceed to centrifugation and resuspension for microscopy.

Fluorescent staining

Timing: 40 min

The cell permeable Hoechst 33342 fluorescent dye in live cells will stain the host nucleus.

11. Gently suspend the dissociated cells in 200 µL of the staining solution and flick the tube to gently mix.

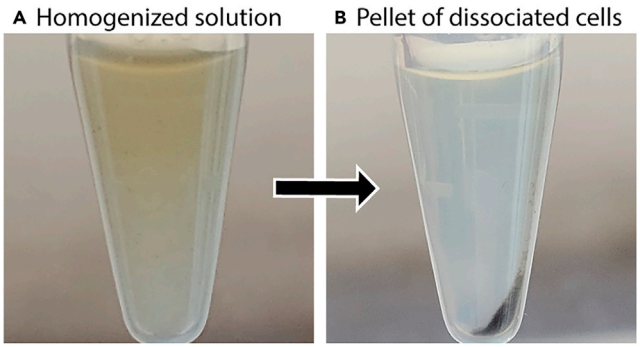

Figure 5. Representative images of dissociated cells following enzymatic digestion and centrifugation
(A) The dissociated Aiptasia cells appear homogenized in the pronase solution following enzymatic digestion.
(B) The dissociated Aiptasia cells are pelleted for the removal of pronase solution and resuspension in staining solution.

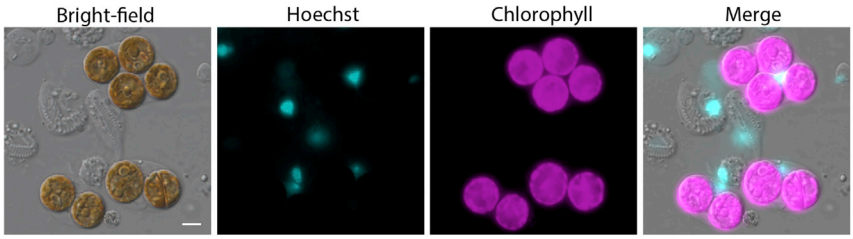

Figure 6. Representative microscopy images of CC7 Aiptasia cells after dissociation depicting symbiotic host cells enclosing *B. minutum* algae and symbiont-free Aiptasia cells

Cyan, cell-permeable Hoechst 33342 fluorescent nucleic acid stain; magenta, algal chlorophyll fluorescence. Scale bar, 5 μm.

12. Incubate the cells for 30 min in the dark at 25°C.
13. Centrifuge the cells at 70 × *g* for 5 min. Remove and discard the supernatant (staining solution).
14. Resuspend the cells in 8–24 μL of CMF-ASW+EGTA.

Mounting and microscopy

Timing: 0.5–1 h

For observation under a microscope, the cell suspension can be mounted on a microscope slide.

15. Take 8–10 μL of the cell suspension with a pipette onto a microscope slide.

Note: It is recommended to use a larger tip, such as a 20 μL vs. a 10 μL, to avoid damaging the host cells during the transfer to the slide.

16. With tweezers, slowly lower a 22 mm × 22 mm coverslip over the droplet until flat, avoiding bubbles.
17. To avoid evaporation of the mounting media, seal the coverslip to the slide using clear nail polish or an alternative sealer.
18. Allow the polish or sealer to dry, then view the slide on a fluorescence microscope. Troubleshooting 5.

Note: We use the Zeiss Axio Observer inverted microscope with ZEN software and 63× oil immersion objective either in bright-field mode or Texas Red filter set for chlorophyll fluorescence (546/12 nm excitation, 590 nm long-pass fluorescence emission), and fluorescence filter set for Hoechst 33342 fluorescent nucleic acid stain (365 nm excitation, 445/50 nm band pass fluorescence emission).

Note: Images can be analyzed and merged using ImageJ.

EXPECTED OUTCOMES

The typical dissociation of adult *Exaiptasia diaphana* tissue results are shown in Figure 6 and 7. The algal chlorophyll fluorescence is used to identify symbiotic cells in combination with the presence of host nuclei, stained with Hoechst 33342. The host membrane can easily be observed using differential interference contrast (DIC) imaging and is useful to further confirm that observed algae are encompassed within the host membrane. The presence of host nuclei between the algae and host membrane indicates that these are intact host gastrodermal cells.

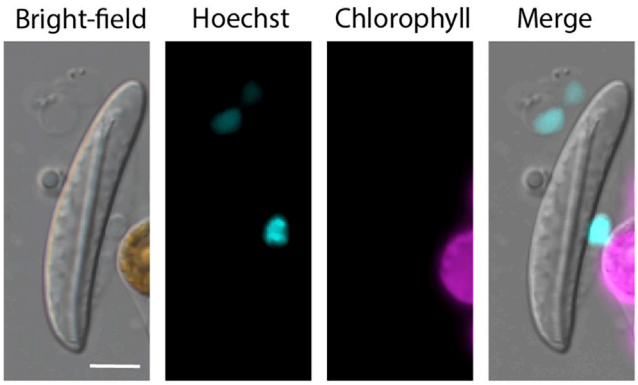

Figure 7. Representative microscopy images of CC7 Aiptasia cells after dissociation depicting an intact host nematocyst cell and symbiont-free Aiptasia cells

Cyan, cell-permeable Hoechst 33342 fluorescent nucleic acid stain; magenta, algal chlorophyll fluorescence. Scale bar, 5 μm.

LIMITATIONS

This protocol uses enzymatic digestion to quickly isolate a single-cell suspension for immediate use. Prolonged cell viability is not optimized in this protocol and the dissociated cells are prone to degradation over time. It is recommended to view the dissociated cells under the microscope or process them for desired analyses as soon as possible post-dissociation.

TROUBLESHOOTING

Problem 1

To wash the animal of external debri and mucus, the animal should be drawn into a transfer pipette and washed by pipetting up and down. Some animals may be too large to fit into the bore size of a transfer pipette (step 1).

Potential solution

Cut the transfer pipette to increase the bore size. Use this wider opening to wash the animal by pipetting up and down. To transfer the animal between wells using the suction of the pipette and to limit the transfer of washing media, return to an uncut transfer pipette.

Problem 2

Mucus is produced by many aquatic organisms for the purpose of enhancing attachment to substrate and protection from factors such as water loss, bacterial infections, and ingestion from predators through its distasteful nature and antibacterial properties.[4,5] In this protocol, mucus can be produced in the transferring and washing steps, which can coat the animal tissue. This can interfere with dissecting the animal tentacles and enzymatic digestion steps (step 3).

Potential solution

Use as little liquid as possible when transferring the animal to the petri dish. Then, using the razor blade and p200 pipette, hold the body of the animal down with the pipette suction while dragging the mucus away from the working area with the razor blade. The mucus should stick together and detach from the animal.

Problem 3

Mucus may be present in the supernatant, making it difficult to remove liquid without disturbing the tissue pellet. It is important to remove the supernatant at this stage to avoid diluting the final working concentration of pronase in the enzymatic digestion (step 5).

Potential solution

- Repeat the centrifugation and pipette the supernatant away from the tissue pellet slowly.
- Move the tissue pellet using a p200 pipette to the top of a 1.5 mL tube. The liquid will then pool at the bottom of the tube, where it can quickly be removed with the p200 pipette.

Problem 4

Animal size and degree of mechanical dissection can impact the dissociation. Chunks of undissociated tissue may remain after enzymatic digestion (step 9).

Potential solution

- Ensure that tentacles have been well-chopped before pronase treatment.
- Gently mix the tissue and pronase mixture two times throughout the pronase incubation by pipetting gently or flicking the tube.
- Increase the length of pronase incubation.
- Increase the final concentration of pronase to 1% (w/v) in CMF-ASW+EGTA.

Problem 5

The following reasons may result in low number of intact host cells (step 18):

- The concentration of pronase is too high or the time of incubation is too long.
- The centrifugation speed was too fast or too long.
- The Aiptasia was small in total diameter (<1 mg tentacle mass).

Potential solution

- Reduce the concentration of the pronase solution to 0.25% with CMF-ASW+EGTA and add Hoechst stain directly to the pronase solution to a final concentration of 20–30 μg/mL. This will reduce the total time of the protocol by combining digestion and staining steps, allowing host cells to be viewed before degradation.
- Ensure that centrifugation speed is below 100 × *g* and no longer than 5 min for each spin.
- For smaller animals, a shorter incubation time is needed, e.g., 5–10 min. Additionally, digestion and staining steps can be combined to reduce overall protocol time.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Andrea L. Kirk (akirk11@uncc.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze any datasets/code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

T.X. conceived the research. A.L.K. performed all experiments. A.L.K. and T.X. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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