Chapter 14

Studying Echinodermata Arm Explant Regeneration Using *Echinaster sepositus*

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Abstract

Echinoderms are marine invertebrate deuterostomes known for their amazing regenerative abilities throughout all life stages. Though some species can undergo whole-body regeneration (WBR), others exhibit more restricted regenerative capabilities. Asteroidea (starfish) comprise one of the few echinoderm taxa capable of undergoing WBR. Indeed, some starfish species can restore all tissues and organs not only during larval stages, but also from arm fragments as adults. Arm explants have been used to study cells, tissues and genes involved in starfish regeneration. Here, we describe methods for obtaining and studying regeneration of arm explants in starfish, in particular animal collection and husbandry, preparation of arm explants, regeneration tests, microscopic anatomy techniques (including transmission electron microscopy, TEM) used to analyze the regenerating explant tissues and cells plus a downstream RNA extraction protocol needed for subsequent molecular investigations.

Key words Echinoderms, Starfish, Regeneration, Arm explants, Transmission electron microscopy, Semithin and ultrathin sectioning, TEM grid staining, RNA extraction

1 Introduction

Echinodermata is a well-known phylum of benthic marine deuterostome invertebrates that includes sea lilies, starfish, brittle stars, sea urchins, and sea cucumbers. Echinoderms and vertebrates belong to the same super phylum named deuterostomes, which includes the chordates and therefore vertebrates, and for this reason share common ancestral traits that were retained during evolution in both lineages. This makes them relevant alternative non-vertebrate models to study potential shared biological mechanisms/processes or to study loss of specific "functions" (e.g., remarkable regenerative abilities, including whole-body regeneration—WBR) along the different evolutionary lineages. Echinoderms have developed phylum-specific morphological

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features, such as secondary radial symmetry, pentameric organization, a miniaturized and modular hydraulic system, that is, the water vascular system, a calcareous endoskeleton (stereom structure), and mutable connective tissues, namely, collagenous tissues that can rapidly modify their intrinsic mechanical properties under nervous system control [1]. Echinoderms are very common and distributed worldwide at almost all depths and latitudes in all marine environments. Indeed, brittle stars can be the dominant macrobenthic fauna on many muddy seafloors and sea cucumbers sometimes account for up to 90% of biomass in the deep oceans [2]. Successful colonization of such diverse biotopes, despite the presence of different predators, may be explained by echinoderms' incredible adaptive capabilities, including their ability to regenerate lost body parts after predation. Although regeneration is observed in all echinoderm taxa, asexual reproduction followed by whole-body regeneration (WBR) is much less common. Notably, some starfish species, primarily *Linckia* spp. and *Coscinasterias spp.*, are capable of extensive WBR: the ability to regenerate the entire individual from a single arm [2–4].

Several starfish species (class Asteroidea), such as *Leptasterias hexactis*, *Asterias rubens, Marthasterias glacialis*, and, more recently, *Echinaster sepositus*, represent the most used models for regeneration studies. Molecular analyses have been recently added to the range of well-known morphological approaches, namely, light and electron microscopy, to identify the mechanisms involved in both developmental and regenerative processes [5–10]. While regeneration studies were traditionally performed in animals with distally amputated arms, nowadays we have developed the better controlled model of arm explants. A double-amputated arm explant (an amputated arm reamputated at its distal tip) represents a simplified and easy-to-use model to investigate the regenerative process, including the relevant cells and tissues plus the activity of different regulatory molecules, that is, signaling and transcription factors, involved. Of particular importance is that the regenerative potential of the arm is tested in the absence of any systemic control by the rest of the body, including its supporting metabolic contribution [11–15]. Remarkably, in starfish arm explants, the distal end/tip initiates regeneration following the same stages observed in standard arm-tip regeneration, thus proving the validity of this cultured model. Furthermore, although to a much less extent, both distalization (namely, the regeneration of the distal-most structures (terminal tube foot and ossicle)) and intercalation (the regeneration of new tissues between the terminal differentiated structures and the stump) apparently occur in both the distal tip of the arm explants and the tip of single amputated arms. On the contrary, at the proximal end of the arm explants, only the terminal elements are regenerated (distalization) with no signs of intercalation being detectable [16] (Fig. 1). This can provide information on the ability

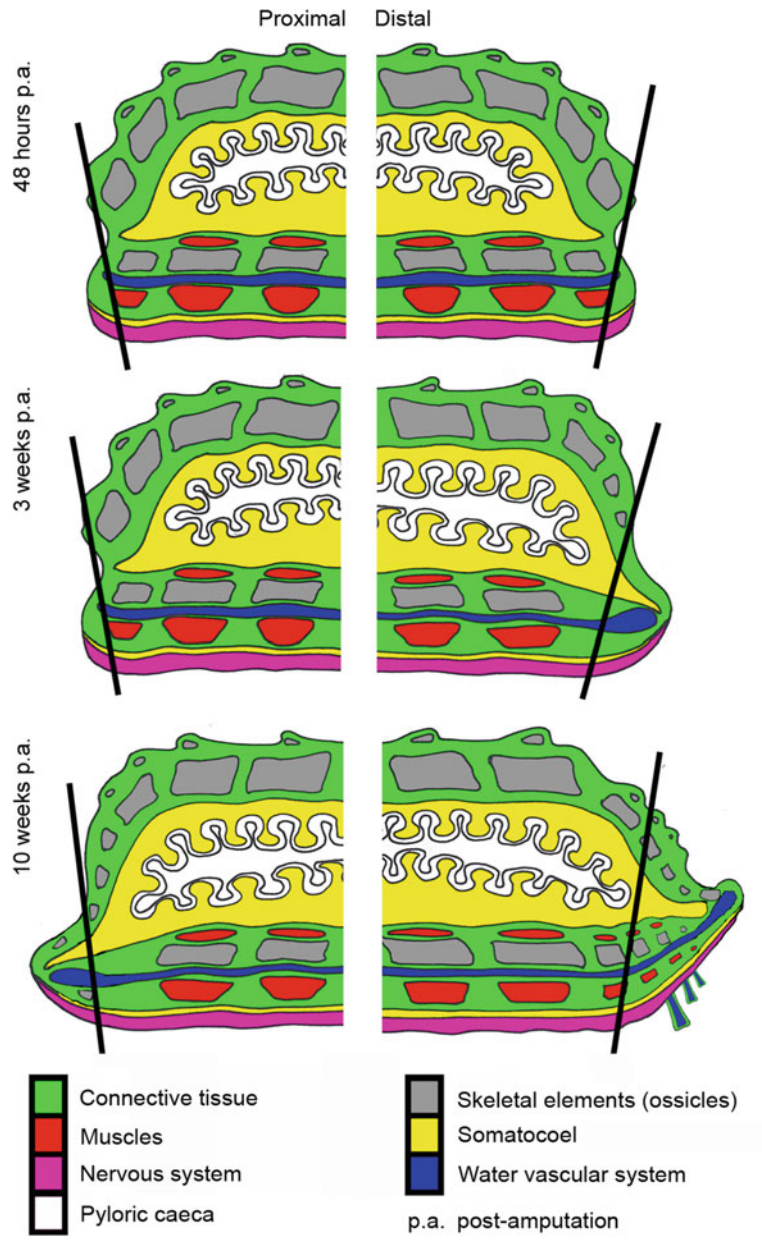


Fig. 1 Longitudinal section schemes of *E. sepositus* arm explants at three selected regenerative stages. For clarity, the tube feet of the nonregenerating arm explant portions have been omitted. Left column: regenerating proximal end. Right column: regenerating distal end. First line: 48 h p.a. Second line: 3 weeks p.a. Third line: 10 weeks p.a. Proximal and distal ends regenerate differently: indeed, while the distal end shows distalization (both terminal ossicle and tube foot) and intercalation (new tube feet), the proximal end shows only distalization (terminal tube foot only) without intercalation. For color coding of tissues, see legend embedded in the figure. Black lines = amputation planes

to "manage" the directionality/polarity of regeneration (i.e., unidirectional vs bidirectional).

Echinaster sepositus, known as the red starfish, is found in the East Atlantic Ocean and in the Mediterranean Sea, where it is one of the most common starfish species. *E. sepositus* inhabits shallow waters, between 1 and 250 m deep, in a wide range of habitats, including rocky and sandy bottoms and seagrass meadows [17]. Its diurnal habits and evident coloration make it clearly visible on any substrate. They live in habitats that are easily accessible and, hence, their collection does not present logistic problems. Its size is sufficiently large to allow easy experimental manipulation and observation of regenerating stages, but still small enough to allow advanced microscopic analyses (e.g., transmission electron microscopy) of the regenerates. Although, as all echinoderms, it is difficult and time-consuming to achieve a full life cycle in the laboratory, adult specimens are rather robust and can be easily maintained in laboratory conditions for long periods (several months or up to 1 year). Overall, these practical features make it a valid and easy-to-handle research model. Indeed, it has been used for many years as model species to study arm tip regeneration using both morphological [18, 19] and molecular [20] approaches. Now we have extended the potential of the species by introducing the culture of arm explants, allowing a more efficient control over the regenerative process. Critical aspects of arm regeneration, such as the control of polarity, the dependence on positional cues, the origin of cells contributing to the different tissues and the regulatory aspects controlling the patterning of newly grown structures, are here more easily studied. In fact, the WBR potential of a single arm or arm piece can be better investigated using arm explants since culturing them allows both better control and easy reproducibility of growing conditions. A seemingly trivial, but extremely useful, characteristic of our model is that, being pentamerous animals, experimentally manipulated and control arm fragments can always be derived from the same animal.

In this chapter, we report methods for collecting and maintaining *E. sepositus* in the laboratory, preparing double-amputated arm explants, and studying the histology/ultrastructure of the regenerative processes using light and transmission electron microscopy (TEM), methods that we complement with those for extracting RNA used for intensive molecular analyses, such as transcriptomics.

2 Materials

All reagents should be prepared using autoclaved filtered distilled water (dH_2O) and kept at room temperature (RT), unless otherwise specified.

2.1 Starfish Collection and Husbandry

1. SCUBA diving equipment: gloves, net.
2. Coolers.
3. Husbandry setup: 50 L glass aquaria, circulation pump, chemical filter (activated charcoal), biological filter (ceramic rings), mechanical filter (filter wool), water oxygenator.
4. Artificial Sea Water (ASW): 37 g commercial marine salts (e.g., Sea Salt, Instant Ocean®) in 1 L deionized water (*see* **Note 1**).
5. Aquarium refrigeration system.
6. Aquarium illumination system with photoperiod control.
7. pH meter.
8. Salinity meter: densimeter or refractometer.
9. Water quality measuring kits: NO_2, NO_3, Cl_2, GH (general hardness), KH (carbonate hardness).
10. Marine oligo-elements (e.g., Marine trace, Seachem).
11. Nitrifying bacteria (e.g., Bactiva, Aquaristica).
12. 1 M $Ca(OH)_2$.
13. Starfish food: 1 cm^3 pieces of squid. Store at −20 °C for up to 1 month.

2.2 Fixation and Epoxy Resin Embedding

Wear gloves and a lab coat and work under a fume hood.

1. 0.2 M sodium cacodylate stock solution: 0.2 M sodium cacodylate trihydrate in dH_2O—pH 7.4 (use drops of 1 M NaOH to adjust pH).
2. 0.1 M sodium cacodylate buffer: 1:1 (v:v) 0.2 M sodium cacodylate stock solution in dH2O. Use a freshly prepared solution, store at 4 °C.
3. Fixative solution: 2% (v/v) glutaraldehyde, 1.4% (w/v) NaCl, in 0.1 M sodium cacodylate buffer (*see* **Note 2**). Use a freshly prepared solution, store at 4 °C.
4. Postfixative solution: 1:1:2 (v:v:v) 4% (v/v) osmium tetroxide, dH_2O and 0.2 M sodium cacodylate stock solution. Osmium tetroxide is highly toxic and must be handled with care. Use a freshly prepared solution, store at 4 °C in darkness.
5. Decalcifying solution: 1:1 (v:v) 4% (w/v) L-ascorbic acid with 0.6 M NaCl. Use a freshly prepared solution, store at 4 °C in darkness.
6. UA solution: 2% (w/v) uranyl acetate in 25% ethanol (EtOH). Uranyl acetate is radioactive and must be handled with care (*see* **Note 3**). Use a freshly prepared solution, store at RT in darkness.
7. Propylene oxide (commercially available solution). Allow to equilibrate at RT before use. Store at 4 °C (*see* **Note 4**).

8. Propylene oxide–Epoxy resin mixture solutions: mix propylene oxide and Epoxy resin in different proportions: 3:1, 1:1, and 1:3. Use a vortex to mix the different solutions and wait until small air bubbles have completely disappeared.
9. Epoxy resin: Epon Araldite-812 (e.g., Fluka-Merck) four components (called A/M, B, C, and Epon). Mix under a fume hood at RT 10 mL A/M, 30 mL B, 12 mL Epon, and 0.8 mL C. Gently mix, avoiding formation of air bubbles, until the resin reaches a homogeneous orange color and honey-like consistency (*see* **Note 5**). Aliquot, store for long term at −20 °C and for short term at 4 °C. Allow to equilibrate at RT under a fume hood before use.
10. Stereomicroscope.
11. Large (at least 1 cm^3) labeled embedding molds (*see* **Note 6**).

2.3 Semithin and Ultrathin Sectioning and Staining

1. Sodium methoxide.
2. 100% methanol (MeOH).
3. 100% EtOH.
4. Crystal violet stain: 1% (w/v) crystal violet in dH_2O, add few drops of 100% EtOH to help dissolving the powder.
5. Basic fuchsin stain: 1% (w/v) basic fuchsin in dH_2O, add few drops of 100% EtOH to help dissolving the powder.
6. Embedding medium (e.g., Eukitt®).
7. 1% (w/v) uranyl acetate in dH_2O (*see* **Note 3**).
8. Lead citrate solution: 1.33 g $Pb(NO_3)_2$, 1.76 g $Na_3(C_6H_5O_7)$ $\bullet 2H_2O$ in 30 mL dH_2O. Shake vigorously for 1–2 min. After 30 min, add 8 mL 1 N NaOH and dH_2O to a final volume of 50 mL. Mix by inversion. Faint turbidity, if present, can be removed by centrifugation.
9. Ultramicrotome.
10. Heater.
11. Light microscope.
12. Disposable glass knife with plastic knife boats (fixed with wax).
13. Glass pipette with round tip (prepared rounding the tip on a flame).
14. Eyelash tool: pull one eyelash, insert it in a glass pipette tip, fix it with a drop of melted wax.
15. Circle tool: prepare a small copper circle (diameter around 3 mm), insert it in a glass pipette tip, fix it with a drop of melted wax.
16. TEM grids (*see* **Note 7**).
17. TEM grid storage box.

2.4 RNA Extraction

1. TRIzol (e.g., Thermo Fisher).
2. Chloroform.
3. RNA *later* (e.g., Thermo Fisher)/liquid nitrogen.
4. Isopropanol.
5. RNase-free water.
6. 75% EtOH in dH_2O.
7. Handheld homogenizer (Pellet Pestle Motor).
8. Pellet pestles.
9. Refrigerated centrifuge.
10. Microvolume spectrophotometer (e.g., NanoDrop, Thermo Fisher Scientific). Agilent 2100 Bioanalyzer System.

3 Methods

3.1 Starfish Collection and Husbandry

1. SCUBA dive in an unpolluted marine area inhabited by *Echinaster sepositus*.
2. Identify a healthy adult specimen (10–12 cm in diameter).
3. Transfer the animal using SCUBA gloves into an appropriate SCUBA net.
4. Repeat **steps 2** and **3** to collect other specimens (see **Note 8**).
5. Transfer the animals into coolers half-filled with fresh seawater (see **Note 9**).
6. Transport them to the laboratory/aquaria facility within the next 2–3 h (see **Note 10**).
7. Add the same volume of ASW as there is natural seawater in each cooler.
8. Wait 2 h for the starfish to acclimate to both the ASW and the new temperature.
9. Transfer 4–5 animals per husbandry setup.
10. Set the photoperiod at 12 h:12 h light–dark.
11. Feed each animal by placing a piece of starfish food (e.g., squid pieces) directly in its mouth.
12. Check that the ASW temperature is about 17 °C.
13. Check that salinity is between 37 and 38‰.
14. Check that pH is between 8–8.2 (see **Note 11**).
15. Use the water quality measuring kits to monitor the chemical parameters of the husbandry systems.

16. Replace half of the aquarium ASW volume in each system with new ASW if NO_2 and NO_3 levels are higher than 0 mg/L and 100 mg/L, respectively.
17. Add nitrifying bacteria elements following manufacturer's instructions.
18. Add marine oligo-elements following manufacturer's instructions.
19. Repeat **steps 11** to **18** for a second week of acclimatization.
20. Continue the maintenance following the same schedule until the end of the experiments.

3.2 Arm Explants

1. Place a starfish on a flat and hard support for amputation (*see* **Note 12**).
2. Amputate one arm proximally at one third of the arm's length with a razor blade (Fig. 2a).
3. Amputate the isolated arm a second time distally at the level of two third of the arm within the next 5 min (Fig. 2a).
4. Repeat **steps 2** and **3** to double amputate all four other arms of the starfish.
5. Collect the five "central" arm segments (the double-amputated arm explants), about 2 cm long.
6. Place the arm explants in properly labeled glass containers filled with ASW (Fig. 2b).

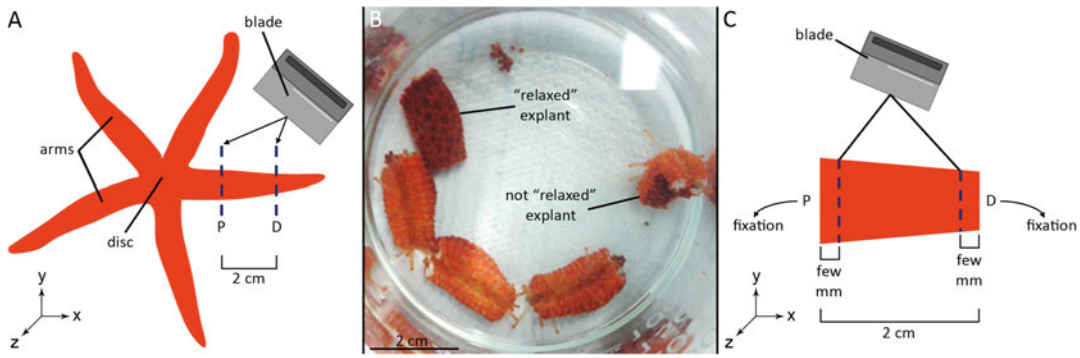


Fig. 2 Starfish arm explant preparation, collection and fixation. (**a**) Top view (*x*–*z*) scheme of *E. sepositus* (aboral side), in which the disc and five slender arms are visible. Using a blade, the central third of each arm is traumatically amputated proximally (P) and distally (D) to obtain five arm explants approximately 2 cm long. For clarity, the amputation of only a single arm is shown. (**b**) After amputation, arm explants are placed back in the aquaria and left to regenerate for the prefixed time-points. They are then collected from the aquaria and placed in glass containers filled with ASW until they are completely "relaxed" (neither curled nor twisted). (**c**) Top view (*x*–*z*) scheme of a "relaxed" regenerating arm explant sectioned using a blade in three portions: proximal, central, and distal. The proximal and distal portions (smaller than the central one) of each explant are fixed and will be processed according to TEM protocols, whereas the central portion can be discharged. *Abbreviations*: D, distal end; P, proximal end. Dashed lines = amputation planes

7. Rapidly place the donor starfish in properly labeled glass containers filled with ASW.
8. Repeat this procedure for all experimental starfish. Use a different glass container for each animal.
9. Do not touch the arm explants or move the container for 30–60 min.
10. Gently transfer the arm explants into the aquaria. Avoid touching the amputated ends and allow them to regenerate over the prefixed time-points (see **step 17**).
11. Keep the five arm explants belonging to the same donor starfish separated from those from other specimens using different aquaria (*see* **Note 13**).
12. Place the donor starfish (with all five arms amputated) in the aquarium with the corresponding arm explants (*see* **Note 14**).
13. During the regeneration period, ASW chemical parameters must be checked and promptly adjusted if necessary (as described in **steps 12–18**, Subheading 3.1).
14. Check and annotate the arm explant health status and behavior.
15. Repeat **step 14** every 2–3 days.
16. Remove dead arm explants (showing connective tissue "melting" or whitish spots on the epidermis) from the aquarium; immediately check ASW parameters (as described in **steps 12–14**, Subheading 3.1) and, if necessary, promptly perform ASW changes to avoid NO_2/NO_3 or pathogen contamination of the whole system (as described in **step 16**, Subheading 3.1).
17. Define at least three regeneration time-points, for example: 48 h post-amputation (p.a.), 3 weeks p.a. and 10 weeks p.a. (see also Fig. 1 and [16, 18, 19]). Based on our experience, arm explants can be successfully maintained in laboratory conditions for 5–6 months.
18. At each prefixed time-point, collect one arm explant from each donor starfish and process them for downstream analyses, both morphological or molecular.

3.3 Fixation for Transmission Electron Microscopy (TEM)

From now on, always wear gloves and a lab coat and work under a fume hood. All solutions should be carefully and gently transferred using clean disposable glass pipettes. All steps should be performed on an orbital shaker (gentle shaking) to facilitate solution penetration and washes. Prepare glass containers with proper labeling for each sample. Before starting, heat the oven at 65 °C. Troubles possibly arising during protocol performance and corresponding troubleshooting are listed in Table 1.

Table 1
Table summarizing common problems arising during microscopy protocol and corresponding troubleshooting

Trouble	Troubleshooting
Poor fixation (possibly due to wrong component concentrations, pH, storage conditions, or contamination) *See* Fig. 4a, b	1. Make sure that the fixative solution is not contaminated (precipitates or debris should not be present) 2. Make sure that the fixative solution has been properly stored, namely, at 4 °C. if so, go to **step 3** 3. Measure the pH of the in 0.2 M sodium cacodylate buffer used to prepare the fixative solution. Adjust to 7.4, using NaOH or HCl depending on recorded value, and use it to prepare a fresh fixative solution 4. Measure the pH of the fixative solution. Adjust to 7.4, using NaOH or HCl depending on recorded value If fixation is still unsatisfactory, prepare fresh fixative solution. Pay particular attention to NaCl concentration and pH. Use the correct ratio of fixative solution per sample volume (1:20). Timing of fixation can be increased up to 2–3 days at 4 °C. Avoid longer fixation to prevent tissue shrinkage
Poor decalcification (possibly due to wrong component concentrations, storage conditions, or contamination) *See* Fig. 4c, d	1. Make sure that the decalcifying solution is not contaminated (precipitates or debris should not be present) 2. Make sure that the decalcifying solution has been properly stored, namely, at 4 °C in darkness If decalcification is still unsatisfactory, prepare fresh decalcifying solution. It is recommended to use larger volumes of solution and change it more frequently to facilitate the decalcification process. Avoid decalcification longer than 3 days at 4 °C in darkness. As suggested, perform the decalcification step on an orbital shaker
Poor dehydration (possibly due to wrong ethanol concentrations or storage conditions)	1. Make sure that the dehydration steps have been properly performed, namely, ethanol has been properly stored and used glass containers have been properly dried before use If dehydration is still unsatisfactory, it is recommended to use larger volumes of ethanol solutions and increase time of each dehydration step. Avoid leaving the samples in direct contact with air during solution changes to prevent tissue rehydration
Poor embedding medium infiltration (possibly due to wrong component concentrations or storage conditions)	1. Make sure that epoxy resin aliquots have been properly stored, namely, well closed, at RT during use, at 4 °C for short-term storage and at

(continued)

Table 1
(continued)

Trouble	Troubleshooting
	−20 °C for long-term storage 2. If epoxy resin does not show the typical honey-like viscosity/consistency and color, prepare fresh epoxy resin and discharge the previous stock If necessary, the proportions of epoxy resin and propylene oxide could be changed to have a more gradual medium infiltration. Perform all steps in the orbital shaker to facilitate embedding medium infiltration
Poor embedding medium polymerization (possibly due to wrong polymerization temperature or timing)	1. Make sure that the temperature of the oven has been properly set, namely, at 65 °C 2. Make sure that the oven has not been opened during the polymerization days 3. Make sure that the oven temperature is stable during the whole polymerization period, namely, 3 days 4. Make sure that polymerization has been performed for the right number of days, namely, 3 If polymerization is still poor, change oven. Note that epoxy resin polymerization can occur also at RT but it will last much longer. Therefore, when possible, prefer polymerization in stable conditions of temperature and humidity that the oven guarantees
Problems with semithin sectioning (possibly due to glass knife damages or ultramicrotome problems). Figure 4e, f	1. Make sure that the glass knife is not compromised. If so, use a new one 2. Make sure that all ultramicrotome parts are well fixed and oriented 3. Make sure that thickness of the section is around 1 μm 4. Make sure that the plastic knife boat is properly fixed to the glass knife, sectioning speed and glass knife orientation are correct in order to avoid on the sample surface the presence of drops that can compromise the sectioning. If so, change it Semithin section thickness and ultramicrotome section speed can be optimized during sectioning. Indeed, both slightly thicker and thinner sections as well as slightly faster and slower section speed can result in better semithin sections. The researcher has to try different combinations to obtain satisfactory results

(continued)

Table 1
(continued)

Trouble	Troubleshooting
Poor semithin section staining (possibly due to wrong solution concentrations or storage). *See* Fig. 4i, j	1. Make sure that sodium methoxide and staining solutions have been properly stored, namely, at RT 2. If debris are present on the glass slides, filter the staining solutions using a syringe and a 0.2 μm filter If staining is still unsatisfactory, both timing of sodium methoxide and staining, and staining solution concentrations can be increased or decreased. Sodium methoxide should be usually increased. Note that differences in staining can be present between samples simply due to sample variability, therefore, the researcher can perform few trials before finding right staining timing and concentrations
Problems with ultrathin sectioning (possibly due to glass knife damages or ultramicrotome problems). *See* Fig. 4g, h	1. Make sure that the glass knife is not compromised. If so, use a new one 2. Make sure that all ultramicrotome parts are well fixed and oriented 3. Prefer using the left side of the glass knife for ultrathin sectioning 4. Make sure that the plastic knife boat is properly fixed to the glass knife in order to avoid on the sample surface the presence of drops that can compromise the sectioning. If so, change it Ultrathin section thickness and ultramicrotome section speed can be optimized during sectioning. Indeed, both slightly thicker and thinner sections as well as slightly faster and slower section speed can result in better ultrathin sections. The researcher has to try different combinations to obtain satisfactory results, namely, ultrathin sections of the following colors: Gold, silver, and white
Poor ultrathin section staining (possibly due to wrong solution concentrations and storage or contamination). *See* Fig. 4k, l	1. Make sure that solutions are not contaminated (precipitates or debris should not be present) 2. Make sure that solutions have been properly stored, namely, UA solution at RT and lead citrate solution at 4 °C 3. If debris are present on the ultrathin sections, filter again the staining solutions using a syringe and a 0.2 μm filter If staining is still unsatisfactory, the researcher can increase the timing of staining, namely, up to 30 min for UA solution and up to 15 min for lead citrate. If so, the following washes in dH_2O can be increased to guarantee the complete elimination of both solutions

(continued)

Table 1
(continued)

Trouble	Troubleshooting
Poor ultrathin section carbon-coating (possibly due to wrong carbon-coating procedure)	1. Make sure that carbon-coating procedure has been properly performed following manufacturer's instructions 2. Perform a second carbon-coating Note that this step of the protocol can be omitted if the transmission electron microscope (TEM) ensures a good level of contrast. Check TEM characteristics with the TEM technician before performing carbon-coating, since it can result unnecessary to obtain micrographs of high quality, in terms of contrast

1. Immediately after collecting the regenerating arm explants, use tweezers to gently transfer them to labeled glass containers filled with ASW.
2. Wait for at least 30 min until they are completely "relaxed"—not curled or twisted, since this can impair fixation and precisely orientated sectioning (Fig. 2b).
3. Gently transfer the arm explants to labeled glass containers filled with fixative solution, avoiding to touch the regenerating tissues with the tweezers.
4. Leave the arm explants in fixative solution for at least 30 min at RT. Place one arm explant on a flat and hard support for amputation (*see* **Note 15**).
5. Rapidly section the arm explant into three portions using a blade: the proximal third, the central third, and the distal third (Fig. 2c) (*see* **Note 16**).
6. Using tweezers, place samples in properly labeled glass containers filled with fresh fixative solution.
7. Discard the central portion.
8. Repeat **steps 3** to **7** for all arm explants.
9. Leave samples in fixative solution for 2 h at 4 °C (*see* **Note 17**). Use a 1:20 volume ratio of fixative solution to sample volume (*see* **Note 18**). Samples can be stored in the fixative solution at 4 °C up to 3 days before further processing.
10. Perform two rapid washes followed by two washes of 15 min in 0.1 M sodium cacodylate buffer to completely remove all traces of fixative solution.
11. Leave samples in the same buffer overnight at 4 °C (*see* **Note 19**).
12. Let samples equilibrate to RT.

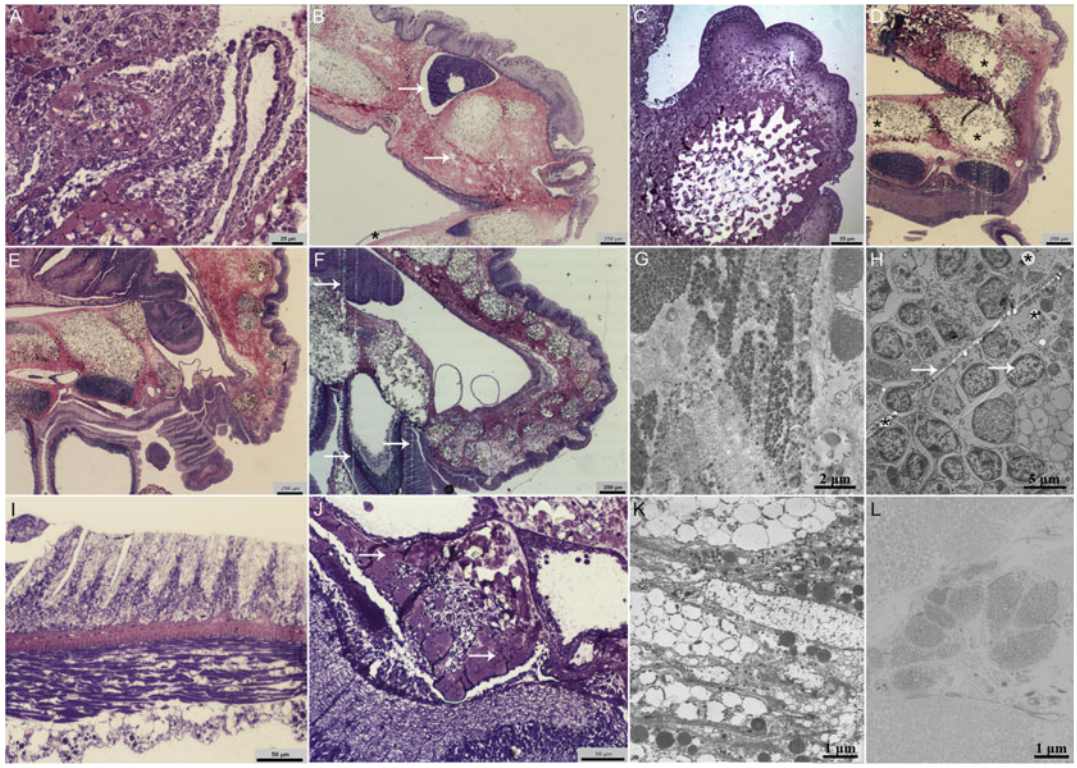


Fig. 4 Example pictures. Light microscopy images (**a**–**f**, **i**, **j**) and TEM micrographs (**g**, **h**, **k**, **l**) showing examples of satisfactory and unsatisfactory results in terms of sample fixation and decalcification, and semithin and ultrathin sectioning and staining. (**a**) Semithin section showing satisfactory sample fixation. All tissues are well preserved. (**b**) Semithin section showing unsatisfactory sample fixation. Artifacts are visible as lacunae and spaces in the tissues (arrows) as well as epithelium detachment (asterisk). (**c**) Semithin section showing satisfactory sample decalcification. The skeletal tissues of a spine, that is, trabeculae are well preserved and no signs of calcium carbonate crystals are visible. (**d**) Semithin section showing unsatisfactory sample decalcification. Skeletal tissues of the ossicles (asterisks) are not well preserved and tissue integrity is therefore lost. (**e**) Semithin section showing satisfactory semithin sectioning. All tissues are well preserved. (**f**) Semithin section showing unsatisfactory semithin sectioning. Vertical lines (arrows) are artifacts due to the sectioning, in particular, to the damaged glass knife edge. (**g**) TEM micrograph showing satisfactory ultrathin sectioning. All tissues are well preserved. (**h**) TEM micrograph showing unsatisfactory ultrathin sectioning. Lines (arrows) and holes (asterisks) are artifacts due to the sectioning, in particular, to the damaged glass knife edge. (**i**) Semithin section showing satisfactory semithin staining. The difference between diversely stained tissues is well visible and different tissue identification is therefore easy and clear. (**j**) Semithin section showing unsatisfactory semithin staining. Basic fuchsin staining (arrows) is not clearly distinguishable from crystal violet staining. Therefore, tissue identification can be more difficult or even wrong. (**k**) TEM micrograph showing satisfactory ultrathin staining. Contrast between electron-dense and electron-lucent cellular elements is well defined. (**l**) TEM micrograph showing unsatisfactory ultrathin staining. Low contrast between electron-dense and electron-lucent portions make it difficult to distinguish among different tissues/cellular elements

13. Remove the 0.1 M sodium cacodylate buffer.
14. Add the postfixative solution.

15. Leave samples immersed in this solution for 2 h at RT in darkness (*see* **Note 20**).
16. Remove the postfixative solution.
17. Perform four gentle washes with autoclaved filtered dH_2O to completely remove traces of osmium tetroxide of the postfixative solution (*see* **Note 21**).
18. Remove dH_2O from the previous wash.
19. Add the decalcifying solution.
20. Leave samples in this solution for at least 24 h in darkness at 4 °C (*see* **Note 22**), changing the solution at least twice a day.
21. Remove the decalcifying solution.
22. Perform several rapid washes in autoclaved filtered dH_2O to completely remove the decalcifying solution.
23. Remove dH_2O from the previous wash.
24. Add 2% (w/v) uranyl acetate in 25% EtOH; leave samples in this solution for 2 h at RT in darkness.
25. Remove the 2% (w/v) uranyl acetate in 25% EtOH.
26. Perform at least 3–4 rapid washes in 25% EtOH to completely remove all traces of the previously used solution.
27. Remove the 25% EtOH from the previous wash.
28. Perform a dehydration in an increasing scale of ethanol: 25%, 50%, 70%, 90%, 95% (two washes of 15 min each), and 100% (three washes of 20 min each) (*see* **Note 23**) at RT. Be sure that samples are never left in direct contact with air, to prevent their rehydration (Table 1).
29. Remove the 100% EtOH from the previous wash.
30. Perform 3–4 rapid washes in propylene oxide at RT.
31. Transfer each of the propylene oxyde-Epoxy resin solutions in the order specified at **step 8** of Subheading 2.2 and leave samples for 1 h in each solution at RT (*see* **Note 24**). When samples are immersed in the first two solutions, glass containers should be left closed; when samples are immersed in the third solution, glass containers should be left partially opened to allow the propylene oxide to evaporate at RT.
32. Remove the remaining 1:3 propylene oxide and Epoxy resin solution.
33. Add pure Epoxy resin and leave samples in pure Epoxy resin overnight at RT under a fume hood.
34. Prepare properly labeled silicon/plastic molds according to the sample size.
35. Add pure Epoxy resin to each mold.

36. Transfer each sample to the corresponding mold using tweezers, avoiding touching the regenerating tissues. During embedding, properly orient samples to facilitate subsequent sectioning. Work under a stereomicroscope positioned under a fume hood, if necessary (*see* **Note 25**).
37. After a final check of the samples' orientation, carefully transfer them to an oven set at 65 °C.
38. Wait 2 days for the resin blocks to polymerize. To avoid changing the temperature and humidity in the oven chamber, do not open the oven during this period (Table 1).
39. Remove the resin blocks from the oven.
40. Store the samples at RT in darkness (*see* **Note 26**).

3.4 Semithin Sectioning and Staining

From now on, always wear gloves and a lab coat.

1. Remove the resin block containing the sample selected for sectioning from the mold.
2. Insert the resin block into the ultramicrotome's sample holder, properly orienting it according to the desired sectioning plane (longitudinal, transverse, frontal, etc.).
3. Fix the resin block tightly to prevent movement during sectioning.
4. Using a blade and working under the stereomicroscope of the ultramicrotome, shape (trim) the resin block to create a pyramid trunk carefully removing excess of resin around the sample (Fig. 3a).
5. Tightly fix the sample holder to the ultramicrotome arm.
6. Fix the glass knife with the plastic boat to the knife stage (*see* **Note 27**).
7. Using a 1 mL syringe add autoclaved filtered dH_2O to the plastic knife boat.
8. Using the proper wheels of the ultramicrotome, orient the knife and the resin block to achieve the desired sectioning orientation.
9. After adjusting the needed parameters of the ultramicrotome, begin sectioning semithin sections of 1 μm in thickness (Fig. 3b). If sectioning is unsatisfactory, the thickness of semithin sections and the speed of sectioning can be modified—a slightly higher thickness than 1 μm and a slower ultramicrotome sectioning speed may result in better semithin sections, considering their quite large square surface (Table 1).
10. Collect semithin sections on properly labeled glass slides (*see* **Note 6**) using a glass pipette. Slowly approach the sections from below, collecting them from below with the glass pipette rounded tip; gently place them in the autoclaved filtered dH_2O drops, prepared using a 1 mL syringe (Fig. 3c).

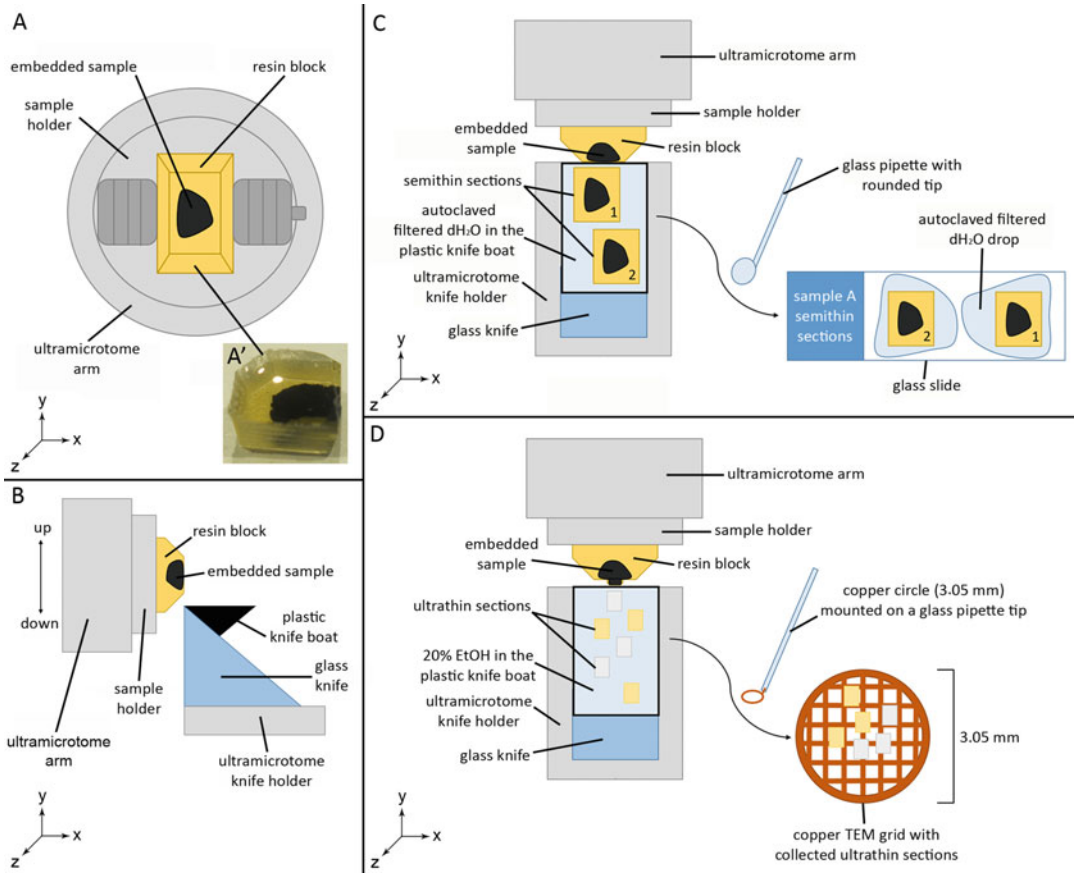


Fig. 3 Procedures to follow for semithin and ultrathin sectioning and semithin and ultrathin section collection. (**a**) Frontal view (*x*–*y*) scheme of the resin block with the embedded sample (black) tightly fixed in the ultramicrotome sample holder, which is positioned in the ultramicrotome arm. (A') Top view (*x*–*z*) image of a resin block with an embedded sample of starfish arm explant. The embedded sample appears black due to postfixation in osmium tetroxide. (**b**) Lateral view (*y*–*z*) scheme of the resin block with the embedded sample (black) positioned in the ultramicrotome arm and of the glass knife (with the plastic knife boat) fixed on the ultramicrotome knife holder. Each time the ultramicrotome arm goes up and down (black arrow on the left), a section (1 μm for semithin sections and 50–90 nm for ultrathin sections) is sectioned by the glass knife and floats onto the liquid within the plastic knife boat, which contains autoclaved filtered dH_2O for semithin sections or 20% EtOH in autoclaved filtered dH_2O for ultrathin sections. (**c**) Semithin section collection. Top view (*x*–*z*) scheme of the glass knife with a plastic knife boat; two semithin sections are floating onto the autoclaved filtered dH_2O inside. Using a disposable glass pipette with a rounded tip, semithin sections can be transferred into autoclaved filtered dH_2O drops on a labeled glass slide (top view; *x*–*z*). (**d**) Ultrathin section collection. Top view (*x*–*z*) scheme of the glass knife with a plastic knife boat; six ultrathin sections are floating onto the 20% EtOH in autoclaved filtered dH_2O inside. Ultrathin sections are much smaller than semithin sections. Using a copper circle mounted on a disposable glass pipette tip, ultrathin sections can be collected from above (maintaining the copper circle parallel to the liquid surface) and positioned on the copper TEM grid (top view; *x*–*z*)

11. Allow the water drops to dry on a heater (70 °C) in a horizontal position.
12. Using a disposable glass pipette place several drops of sodium methoxide on the sections, allowing it to completely remove the resin for at least 30 s.
13. Insert slides into a glass Coplin jar filled with 100% MeOH for 1 min to remove the sodium methoxide.
14. Insert slides into a glass Coplin jar filled with 100% EtOH for 1 min to remove the 100% MeOH.
15. Rapidly wash the slides with tap water followed by autoclaved filtered dH_2O.
16. Allow the dH_2O to dry on a heater (70 °C) in a horizontal position.
17. Using a disposable glass pipette place several drops of 1% (w/v) crystal violet on the sections, allowing it to stain for 30–60 s. The staining time must be optimized according to the type of tissue either changing crystal violet concentration or staining time (Table 1 and Fig. 4).
18. Rapidly wash the slides with tap water and autoclaved dH_2O.
19. Allow the dH_2O to dry on a heater (70 °C) in a horizontal position.
20. Using a disposable glass pipette place several drops of 1% (w/v) basic fuchsin on the sections, allowing it to stain for 15–30 s. The staining time must be optimized according to the type of tissue either changing basic fuchsin concentration or staining time (Table 1 and Fig. 4).
21. Rapidly wash the slides with tap water followed by autoclaved dH_2O.
22. Allow the dH_2O to dry on a heater (70 °C) in a horizontal position.
23. Mount the slides using few drops of Eukitt® and glass coverslips according to the number of sections present on the glass slides.
24. Let the Eukitt® dry for several minutes on the heater (70 °C) in a horizontal position.
25. Observe the slides under a light microscope. If staining is not satisfactory, modify the staining concentrations (both lower and higher than 1% (w/v)), as well as the staining time (both lower and higher than 15–60 s) on the following slide (Table 1 and Fig. 4).
26. Repeat **steps 8** to **25** to continue sectioning, collection, and observation of semithin sections until the desired sample plane with tissues of interest has been reached.

27. Leave mounted glass slides overnight at RT in a horizontal position.
28. Store mounted glass slides indefinitely in proper glass slide boxes at RT in darkness.

3.5 Ultrathin Sectioning

From now on, always wear gloves and a lab coat.

1. After reaching the desired sample plane, performing the semithin sectioning, and checking the tissues of interest under the light microscope, remove the sample holder from the ultramicrotome arm.
2. Place the sample holder on the sample stage.
3. Using a thin blade and working under the ultramicrotome's stereomicroscope, shape (trim) the sample surface to create a small pyramid trunk (less than 1 square mm) only on the desired tissues (*see* **Note 28**).
4. Place the sample holder back on the ultramicrotome arm and close tightly.
5. Using a 1 mL syringe remove autoclaved filtered dH_2O from the plastic knife boat.
6. Using a 1 mL syringe add 20% EtOH in autoclaved filtered dH_2O to the plastic knife boat.
7. After adjusting the needed parameters of the ultramicrotome, begin sectioning ultrathin sections of 50–90 nm in thickness (Fig. 3d). If sectioning is not satisfactory, modify the thickness of ultrathin sections and the speed of sectioning; slightly higher or lower thickness than 50–90 nm and a slower ultramicrotome sectioning speed may result in better ultrathin sections (Table 1 and Fig. 4).
8. Ultrathin sections floating on the 20% EtOH inside the plastic knife boat can be serially and carefully collected on a TEM grid.
9. Only white, silver, or gold ultrathin sections must be collected: indeed, the color of the section indicates the correct thickness (between 50 and 90 nm). Sections that are too thick (more than 100 nm) show a different color, namely, blue, green, or pink.
10. To collect ultrathin sections, use an eyelash mounted on a glass pipette. Do not directly touch the sections with the eyelash. It is better to use the eyelash to move the 20% EtOH around the sections to group them before collection.
11. Decide which side of the TEM grid to use to collect the sections; use the same side for all grids.
12. Using tweezers (without excessive pressure to avoid deformation), pick a TEM grid.

13. Slowly collect all sections inside using a small copper circle by approaching them from above.
14. Maintaining the copper circle parallel to the TEM grid surface, slowly approach the TEM grid and allow the sections to adhere to the grid surface (Fig. 3d).
15. Once sections have been attached to the grid surface, gently remove excess 20% EtOH using a small piece of filter paper.
16. After the grid has completely dried, store it in a TEM grid storage box with proper labeling (*see* **Note 29**).
17. Repeat **steps 7** to **16** to continue sectioning and collecting ultrathin sections of the tissues of interest.
18. If desired, after ultrathin sectioning, some semithin sections can also be collected, stained, and observed (as described in Subheading 3.4) in order to determine if ultrathin sectioning of the tissue of interest is complete.

3.6 Ultrathin Section Staining and TEM Grid Carbon-Coating

From now on, always wear gloves and a lab coat and work under a fume hood for the ultrathin section staining.

1. Insert 1 mL of 1% (w/v) uranyl acetate in a 1 mL syringe covered with aluminum foil to block radiation.
2. Prepare a piece of Parafilm® in a large glass petri dish.
3. Using this syringe with the 0.2 μm filter, prepare a droplet of 1% (w/v) uranyl acetate on the Parafilm® for each grid that must be stained (*see* **Note 30**).
4. Transfer the selected grids face down on the single droplets using tweezers (do not touch ultrathin sections while handling the grids). Be sure that the side of the grids with ultrathin sections is in direct contact with the staining solution. If properly done, the grids will float on the droplets due to surface tension (Fig. 5a).
5. Close the large glass petri dish and cover it with aluminum foil to block radiation.
6. Wait for 20 min.
7. Use a 1 mL syringe with a 0.2 μm filter to prepare (on another piece of Parafilm®) 10 droplets of autoclaved filtered dH_2O for each grid.
8. Use a 1 mL syringe with a 0.2 μm filter to prepare (on another piece of Parafilm®) a droplet of lead citrate solution for each grid that must be stained.
9. In the large glass petri dish where the piece of Parafilm® is positioned, insert 3–4 NaOH pellets in order to keep the glass petri dish dry.

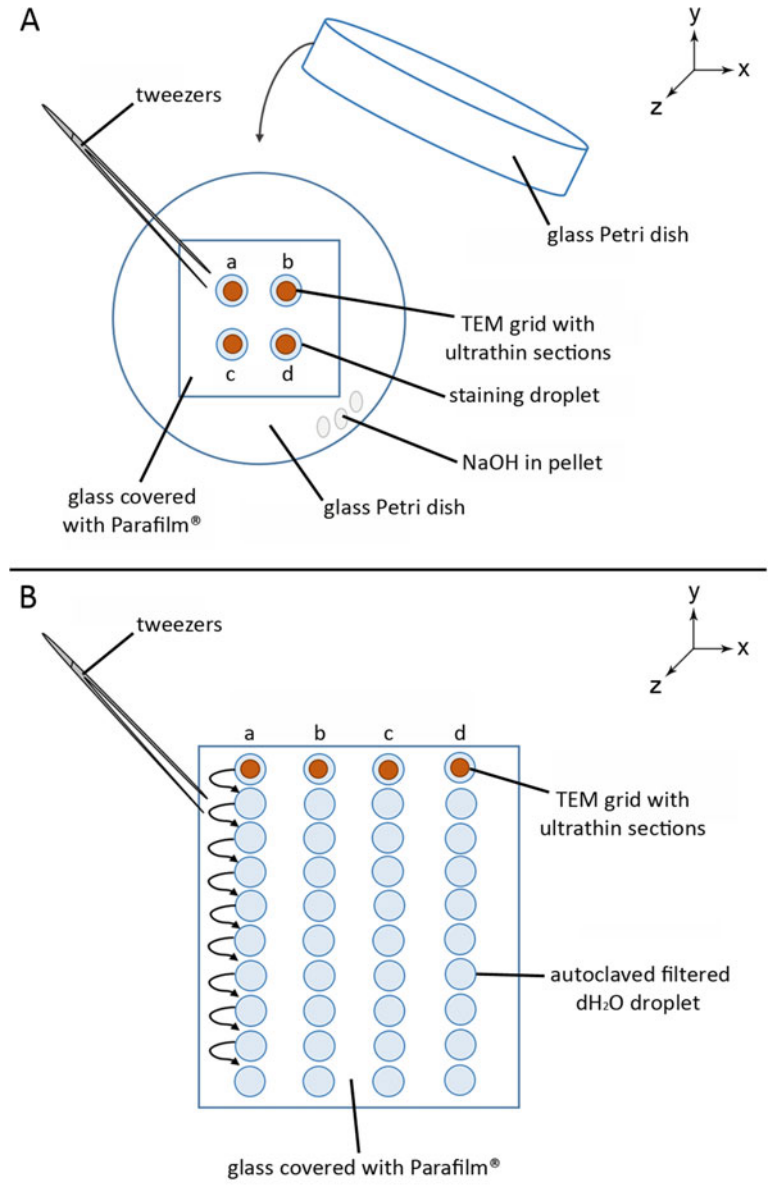


Fig. 5 Ultrathin section staining procedure. (**a**) Top view (*x*–*z*) scheme of TEM grid staining. TEM grids are gently placed in droplets of staining solution using tweezers. The TEM grid position is accurately labeled. When exposed to 1% uranyl acetate, the glass petri dish must be covered with aluminum foil and must not contain NaOH pellets. The latter are needed only in the lead citrate step. (**b**) Top view (*x*–*z*) scheme of TEM grid-washing steps after staining. TEM grids are gently washed in subsequent autoclaved filtered dH_2O droplets, using tweezers (arrows) to completely remove traces of staining solution (1% uranyl acetate or lead citrate). During all steps, ultrathin sections face the liquid. The droplets of various solutions are prepared on a glass covered with Parafilm®, using 1 mL syringes with a 0.2 μm filters. The TEM grid position is accurately labeled

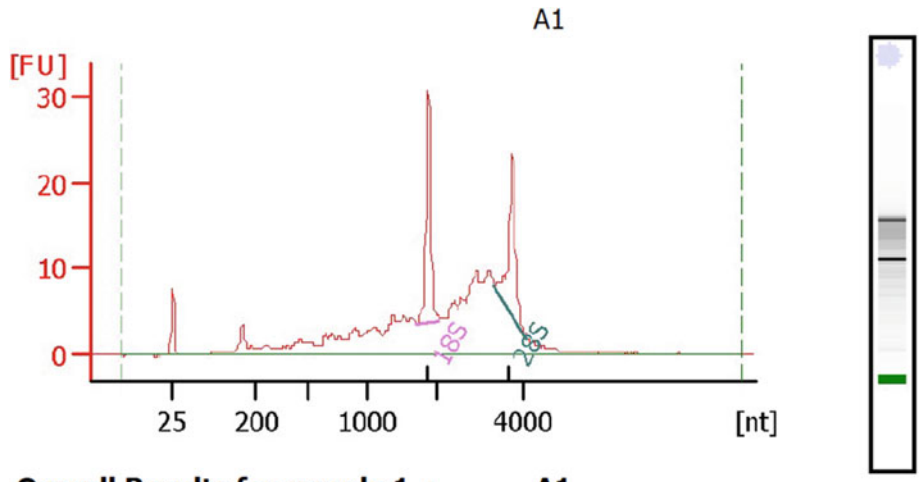
10. Wait until the grids have been in the staining solution for 20 min.
11. Gently using tweezers, sequentially transfer each grid (always face down) to the 10 droplets to rapidly wash the ultrathin sections and eliminate all traces of 1% (w/v) uranyl acetate. If properly done, the grids will float on the droplets due to surface tension (Fig. 5b).
12. When all grids have been washed, gently use tweezers to transfer them (face down) to the single droplets of lead citrate solution. If properly done, the grids will float on the droplets due to surface tension (Fig. 5a).
13. Wait for 8–12 min, depending on the level of contrast needed (Table 1 and Fig. 4).
14. During this time, use a 1 mL syringe with a 0.2 μm filter to prepare (on another piece of Parafilm®) 10 droplets of autoclaved filtered dH_2O for each grid.
15. Gently using tweezers, sequentially transfer each grid (always face down) to the 10 droplets to rapidly wash the ultrathin sections and eliminate all traces of lead citrate solution. If properly done, the grids will float on the droplets due to surface tension (Fig. 5b).
16. Gently dry for few seconds all grids using a small piece of filter paper. Avoid direct contact between the filter paper and ultrathin sections positioning the grid face up on the filter paper.
17. Transfer the grids face up on a properly labeled piece of filter paper.
18. Transfer the grids to a carbon-coater and follow the manufacturer's instructions to coat the ultrathin sections with a thin layer of carbon that will stabilize the ultrathin sections under the TEM beam. This step can be omitted (Table 1).
19. Store the grids in the TEM grid storage box at RT in darkness until observation under TEM.
20. Image the sections using TEM following the manufacturer's instructions.
21. If ultrathin section staining (contrast) results are not satisfactory during observation under TEM, increase the staining times for both 1% (w/v) uranyl acetate and lead citrate (Table 1 and Fig. 4).

3.7 RNA Extraction

The following protocol has been successfully employed to perform RNA extraction from regenerating arm tips of both asteroids and ophiuroids. It has been performed on *E. sepositus* regenerating arm explants, with the same success (*see* **Note 31**). The extraction protocol final aim has been either the cloning of fragments by

conventional PCR [21] or obtaining RNA of high quality for transcriptome analysis [22]. From now on, always wear gloves and a lab coat and work under a fume hood.

1. Using a scalpel, dissect out the regenerating tissues from both sides (proximal and distal) of each regenerating explant, and avoid collection of nonregenerating tissues or stump tissue.
2. Immediately freeze each piece (regenerating tissue) in a properly labeled 1.5 mL tube in liquid nitrogen or in RNA *later* or go to **step 5** to start RNA extraction from fresh tissue.
3. Store samples at −80 °C (liquid nitrogen) or at −20 °C (RNA *later*) for up to several months until use.
4. Remove the samples from the freezer and keep on ice.
5. Add 1 mL of TRIzol per approximatively 100 mg of tissue. Once the TRIzol is added it is not needed to work on ice any more.
6. Homogenize each sample using the Pellet Pestle Motor.
7. Mix the sample by pipetting up and down a few times using a P1000.
8. Add 200 µL of chloroform per each mL of TRIzol added in **step 5**.
9. Close the tube and shake it vigorously for 15 s.
10. Incubate 2–3 min at RT.
11. Centrifuge the homogenate at 11,000 rcf for 15 min at 2–8 °C.
12. Collect the upper aqueous phase and place it into a new properly labeled 1.5 mL tube.
13. Discard the other phases.
14. Add 500 µL of isopropanol to the solution and incubate at room temperature for 10 min.
15. Spin at 10000 rcf for 10 min at 2–8 °C; a gel-like pellet should form at the bottom of the tube.
16. Carefully remove the supernatant.
17. Add 1 mL of 75% EtOH.
18. Vortex for 5 s at RT.
19. Spin at 10,000 rcf for 5 min at 2–8 °C.
20. Carefully remove the supernatant.
21. Air dry the pellet for 5 min at RT.
22. Dissolve the pellet in 50–100 µL of RNase free water (*see* **Note 32**).
23. Quantify and qualify the obtained RNA using a spectrophotometer (*see* **Note 33** and Fig. 6).



Overall Results for sample 1 : A1

RNA Area:	245,7
RNA Concentration:	149 ng/µl
rRNA Ratio [28s / 18s]:	0,9
RNA Integrity Number (RIN):	6.6 (B.02.07)
Result Flagging Color:	
Result Flagging Label:	RIN: 6.60

Fragment table for sample 1 : A1

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1.705	2.076	26,8	10,9
28S	3.329	4.092	23,9	9,7

Fig. 6 Sample electropherogram of RNA extract from regenerating tissue of *E. sepositus*. A total RNA sample is analyzed on the Agilent 2100 Bioanalyzer System using the Eukaryote Total RNA Nano assay. RIN (RNA Integrity Number) software algorithm allows for the quality determination of eukaryotic total RNAs, based on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact

24. Synthesize cDNA following standard procedures for downstream experiments, that is, transcriptome analysis or RNA probe synthesis for in situ hybridization. Samples with highest RIN values should be selected, as they are of the highest quality.

4 Notes

1. ASW should be prepared 2–3 weeks before introducing animals into the aquaria and left to run in aquaria that are completely equipped with all filters. This time is necessary to stabilize the chemical/physical parameters and allow the growth of a sufficient population of nitrifying bacteria. To facilitate this process,

the researcher can add commercially available bacteria useful for both initial set up of the aquarium and long-term maintenance and partial water renewal. In the latter case, ASW should be prepared 2–3 days in advance before adding it to the system.

2. The concentration of NaCl may change depending on the salinity of the seawater where starfish were collected. For example, if the recorded salinity at the collection site is approximately 37‰, 1.4% (w/v) NaCl should be used, whereas at 33‰ salinity, 1.2% (w/v) NaCl is preferred.
3. Only specialized personnel can prepare this solution; waste must be handled and treated as radioactive (e.g., aluminum foil should be used to cover all disposables and materials used in order to block radiations). Before starting the protocol, the researcher must be aware of the risks connected with handling this reagent, verify that authorizations are valid, and strictly follow the instructions of the radioactive laboratory manager.
4. Propylene oxide is highly volatile. Open and use only under a fume hood. Once opened, store at 4 °C. Propylene oxide melts plastic and Parafilm®. Therefore, avoid direct contact with these materials; before use, check if the plastic tubes are resistant to this reagent. If not, use glass tubes instead.
5. This is a crucial step: a nonhomogeneous embedding medium will not perfectly infiltrate the tissues and polymerize (Table 1).
6. Glass containers and plastic disposable materials used during the protocol should be properly labeled using pencils on paper tape, since several of the solvents employed (e.g., EtOH) remove the ink. Prefer pencils also to label the glass slides used to collect the semithin sections.
7. The researcher can choose among many types of TEM grids (usually 3.05 mm in diameter) that vary in terms of materials (copper, nickel, silver, gold, etc.), meshes (50, 100, 150, 200, 300, 400, etc.), and cost. For standard TEM analyses, copper grids with meshes from 200 to 400 are generally preferred.
8. Avoid pressing and crowding of the starfish during collection.
9. A good water–animal's ratio is about 10 L per starfish.
10. Since *E. sepositus* has a delicate epidermis, avoid shaking the animals during transportation. Moreover, avoid abrupt changes in seawater temperature during transport. Ice packs can be added to the coolers to help maintain the same temperature as that of the sampling site. Starfish tolerate these transport conditions for several hours.
11. Organic materials (uneaten food, feces, etc.) can cause a decrease in sea water pH. This can be adjusted using drops of 1 M $Ca(OH)_2$.

12. This step should be completed in "dry" conditions. The animals should not be immersed in ASW to facilitate specimen handling.
13. One large aquarium provided with proper internal separators can be used as well.
14. The donor animals will regenerate. Note that after some days, the arm explants may begin moving throughout the aquarium.
15. This step should be completed in "dry" conditions. The arm explants should not be immersed in ASW to facilitate specimen handling.
16. Both proximal and distal portions should be large enough to avoid tissue deformation/rupture when sectioned with the blade but should not exceed 5–7 millimeters in thickness, to allow the fixative solution to penetrate all tissues. Use a different blade for each arm explant.
17. The researcher can leave samples in the fixative solution for a maximum of 2–3 days at 4 °C, to prevent shrinkage of the tissues.
18. To facilitate fixative penetration, a glass pipette can be used to gently insert the fixative solution into the perivisceral coelomic cavity (somatocoel), where tissues, such as the pyloric caeca (*see* Fig. 1), may partially block fixative solution infiltration into deep tissues.
19. If samples are small and the researcher must shorten the protocol times, the researcher can also leave the samples in 0.1 M sodium cacodylate trihydrate buffer for just 3–4 h and then proceed with the following protocol step.
20. When samples contact the postfixative solution, due to the presence of osmium tetroxide, they rapidly turn black. After 2 h, check that samples are totally black to be sure that all tissues have been properly postfixed.
21. The researcher should also carefully wash (with dH_2O) the inner walls of the glass containers where samples are immersed, since small crystals of osmium tetroxide can cause artifacts under TEM.
22. Depending on the sample size, the researcher can leave samples in decalcifying solution for a maximum of 3 days (at 4 °C in darkness). Large volumes are preferable to facilitate the decalcification process. Therefore, if necessary, use tweezers to gently transfer samples in labeled glass containers bigger than those used for the previous steps.
23. The researcher can stop the protocol when samples are immersed in 70% EtOH and leave them at 4 °C in darkness for several weeks. In this case, glass containers completely filled

with 70% EtOH should be tightly closed with Parafilm® to prevent EtOH evaporation.

24. If the samples are particularly small, the researcher can skip the steps involving the 3:1 propylene oxide and Epoxy resin, and the 1:3 propylene oxide and Epoxy resin, instead performing only the 1:1 propylene oxide and Epoxy resin step for at least 2 h.
25. Because the samples are black, we recommend orienting the samples with the oral side face down in the mold (tube feet are always easily recognizable) and properly labeling the mold so that it will be easy to distinguish the arm explant proximal and distal ends.
26. Samples embedded in Epoxy resin can be stored at RT in darkness for at least several years, virtually forever.
27. The researcher should consider that semithin sections will be several millimeters wide; therefore, a glass knife wider than the sample width must be used to avoid problems during sectioning. Fix the knife tightly to prevent it from moving during sectioning; adjust the knife-stage inclination degrees.
28. The researcher should be careful in shaping (trimming) the small pyramid trunk with the thin blade to avoid damage to the samples, especially the tissues of interest. Moreover, when possible, the researcher should avoid including skeletal (calcitic/mineralized) tissues, such as ossicle or spines in the small pyramid trunk—even after the decalcifying step, microscopic traces of calcium carbonate crystals may remain present in these tissues and impair sectioning, ruining the blade edge. If skeletal elements are the tissues of interest, the researcher should be aware that the edges of glass knives may be damaged. Therefore, they must be frequently changed to obtain proper ultrathin sections. The edges of diamond knives may be permanently damaged, with consequent high costs for repair and/or new purchase.
29. TEM grids can be stored in the TEM grid storage box virtually forever at RT in darkness.
30. The researcher must properly label the position of each grid on the piece of Parafilm®, especially if grids belong to different samples.
31. At this stage no specific results are reported since the isolated RNA can be processed for different analyses, that is, transcriptomics or cDNA synthesis.
32. Optional step: heat the tube to 55 °C for 10 min to help dissolve the RNA.
33. The quality of extracted RNAs is routinely tested in an Agilent 2100 Bioanalyzer System. Measures of integrity are given as

RIN (RNA integrity number) values. RINs are calculated based on the mobility run of an RNA sample through a capillary electrophoresis.

Acknowledgments

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