# Chapter 4

# Studying Porifera WBR Using the Calcerous Sponges *Leucosolenia*

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## Abstract

Sponges (Porifera), basal nonbilaterian metazoans, are well known for their high regenerative capacities ranging from reparation of a lost body wall to whole-body regeneration from a small piece of tissues or even from dissociated cells. Sponges from different clades utilize different cell sources and various morphological processes to complete the regeneration. This variety makes these animals promising models for studying the evolution of regeneration in Metazoa. However, there are few publications concerning the regenerative mechanisms in sponges. This could be partially explained by the delicacy of sponge tissues, which requires modifying and fine adjusting of common research protocols. The current chapter describes various methods for studying regeneration processes in the marine calcareous sponge, *Leucosolenia*. Provided protocols span all significant research steps: from sponge collection and surgical operations to various types of microscopy and immunohistochemical studies.

**Key words** Regeneration, Sponges, Protocols, Microscopy, Surgical operations, Ultrastructure, Proliferation, Apoptosis

## 1 Introduction

Sponges (Phylum Porifera) are thought to be the sister group of all other animals and the earliest branching multicellular lineage of extant animals [1]. As such, they represent a key group for the understanding of the evolutionary history of animals, including the origin and evolution of regeneration mechanisms. The body shape of sponges is very diverse; they may be film-like, encrusting, lumpy or spherical, tubular, branching, flabellate, and so on. The body size of sponges varies as much as their body shapes: from 3–10 mm to 1.5–2 m [2]. Their organization is peculiar: they have no distinct gut, muscles, gonads, nervous system, or respiratory system. The surface of a sponge is covered by a simple single-layered flat epithelium (called exopinacoderm), while the internal parts of the animal body are occupied by a highly complex mesenchymal tissue (called mesohyl) that comprises numerous mobile cell

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types embedded in its extracellular matrix [3]. The rigidity of the sponge body is ensured by the collagen and spongin fibrils (in some Demospongiae orders) and by the internal inorganic skeleton, consisting of either calcium carbonate ($CaCO_3$) (Calcarea, some Demospongiae) or silica ($SiO_2$) (Hexactinellida, Demospongiae, many Homoscleromorpha).

The mesohyl is penetrated by a complex system of canals and choanocyte chambers (termed the aquiferous system), which is the most characteristic feature of the poriferan anatomy. Sponges use this constant water pumping system to obtain food and oxygen and to remove metabolic wastes. Surrounding water is drawn into the inhalant canals via numerous pores (ostia) in the exopinacoderm. Water then circulates through choanocyte chambers, where it is filtered, before leaving the sponge via the system of exhalant canals that converge to large exhalant openings (osculum) [4]. The choanocyte chambers are lined by flagellated collar cells (called choanocytes). The constant beating of choanocytes' flagella generates the water flow through the whole aquiferous system, and their collars serves as an ultimate filter for retaining particles from the pumped water [2].

Five types of the body organization have been described in sponges: (1) asconoid, (2) solenoid, (3) syconoid, (4) sylleibid, and (5) leuconoid [3]. These types differ by the complexity of the aquiferous system and the extent of the mesohyl development. In the simplest asconoid sponges, ostia lead directly to a single cavity completely lined with the choanocytes, and the mesohyl is represented only by thin, mostly acellular layer. The more complex leuconoid sponges are characterized by an elaborated aquiferous system with highly developed canals and numerous choanocyte chambers and thick mesohyl with numerous specialized cell types.

A characteristic feature distinguishing sponges from other Metazoa is the high plasticity of cellular differentiation, anatomical, and tissue structures throughout their life cycle. Various differentiated cells of the sponge can move, transdifferentiate, and switch functions. The direction of the differentiation depends on the current needs of the organism. Thus, the sponge is constantly in the state of rearrangements of all its structures [5–9]. This "chronic morphogenesis" contributes to the growth of the animal, for instance, by reconstructing its somatic tissue after degradation during sexual and asexual reproduction, as well as during regeneration [10–13]. Besides, sponges are not equipped with protective tissues or structures like cuticles, scales, or shells, but are covered only by a single-cell layer. It has been suggested that this lack of protection against injury closely correlates with the high regenerative capacity of sponges [14].

Sponges are known to possess remarkable reconstitutive abilities ranging from restoration of a lost body part to a complete organism development from a small piece of tissue and even from

the cell suspension. However, only few reliable data on the regeneration mechanisms (morphogenesis, cell behavior, and regulation) and their distribution among sponge clades currently exists [10, 12, 15–18].

We provided complex and detailed investigations of reparative regeneration in homoscleromorphs [10], calcareous sponges [13, 19], and demosponges [11, 12]. These studies included various approaches: transmission electron microscopy (TEM), scanning electron microscopy (SEM), epifluorescent and light microscopy, immunohistochemistry, and time-lapse recordings. The obtained results show a high diversity of morphogenesis, cell mechanisms, and cell turnover, accompanying the regeneration processes.

The model sponges presented in this chapter belong to the genus *Leucosolenia*, an abundant species broadly distributed in the White Sea and in the North of Europe, where they are accessible throughout the year. *Leucosolenia* are calcareous sponges, characterized by a calcium carbonate mineral skeleton and the asconoid aquiferous system (Fig. 1a).

The body wall of *Leucosolenia* has a thickness of 15–30 μm and is composed of three layers: an outer layer—the exopinacoderm, a central region—the loose mesohyl, and an inner layer—the choanoderm (Fig. 2b). Inhalant pores (ostia) are scattered throughout the exopinacoderm. They are formed by tubular cylindrical cells (porocytes), which connect the external milieu with the internal choanocyte cavity. The mesohyl of *Leucosolenia* contains a variety of

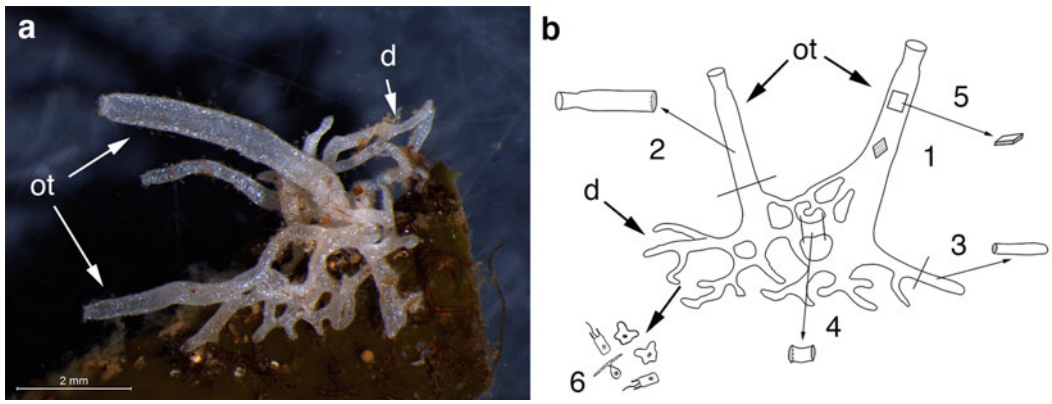


**Fig. 1** Surgical operations in *Leucosolenia variabilis*. (**a**) sponge in vivo; (**b**) scheme of a sponge with different types of surgical operations: (1) body wall regeneration, (2) whole-body regeneration (WBR) from an amputated oscular tube, (3) WBR from an amputated diverticulum, (4) WBR from an amputated cormus tube, (5) WBR from a small fragment of the body wall, (6) cell reaggregation after mechanical tissue dissociation. WBR could be observed during the restorative process in amputated body tubes (2–4), small fragments of the body wall (5) and during cell reaggregation after dissociation (6). WBR from amputated body tubes requires minimal rearrangements of intact tissues, while cell reaggregation is accompanied by complete destruction of intact tissue structure. WBR from small fragments of the body wall represents an intermediate type. *d* diverticula, *ot* oscular tubes

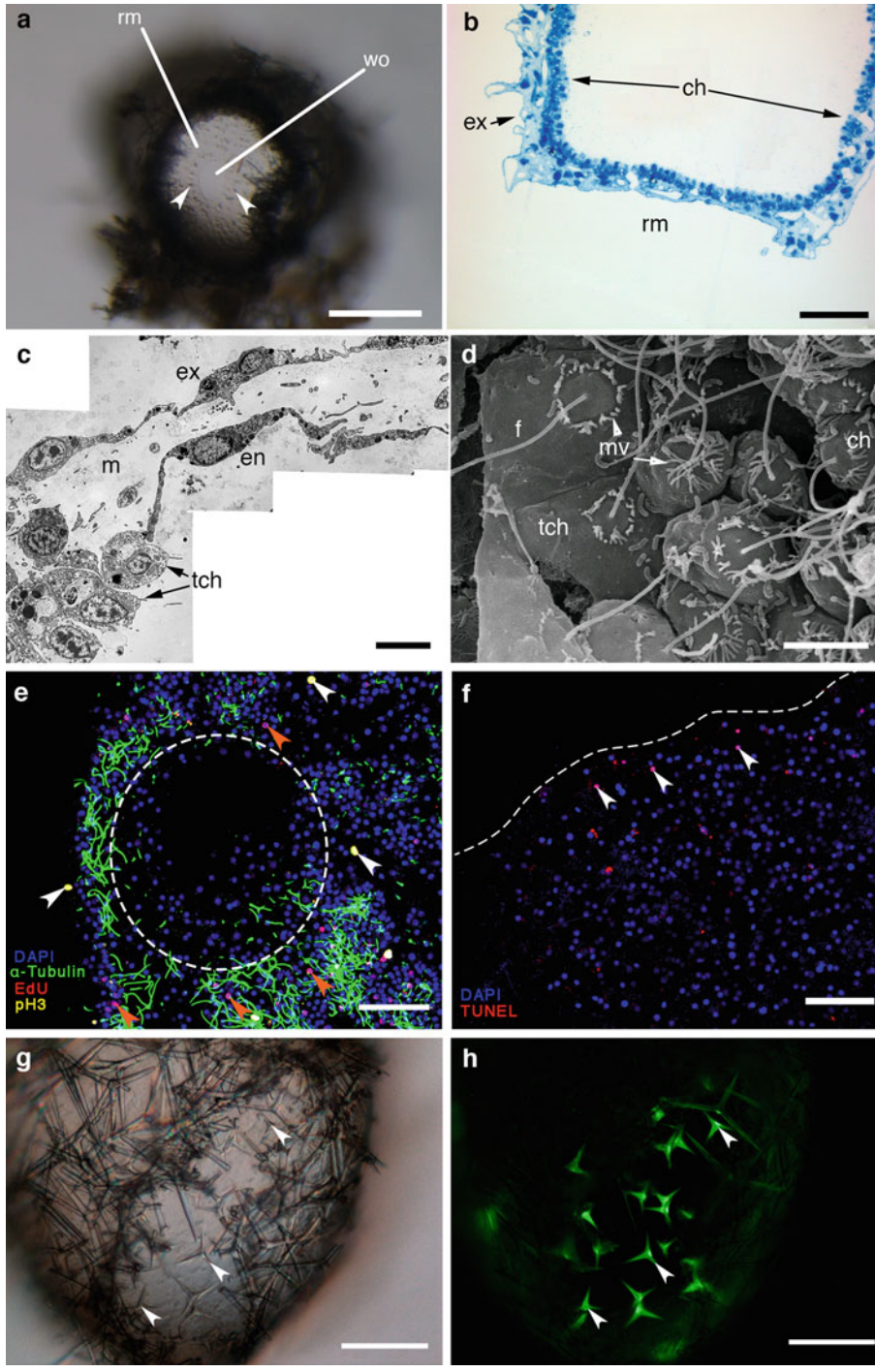


**Fig. 2** Various methods for investigations of *Leucosolenia variabilis* regeneration. (**a**) regenerative membrane growing from the periphery to the center of the wound orifice, stereomicroscope (in vivo), white arrowheads mark mesohyl cells inside the regenerative membrane. (**b**) Semithin section of regenerative membrane transformed into intact body wall (96 hpo). (**c**) TEM micrograph of complete regenerative membrane (24 hpo) consisting of the flattened exopinacocytes and endopinocytes transdifferentiated from choanocytes. (**d**) SEM micrograph of choanocytes transdifferentiating into endopinacocytes during growth of regenerative

cell types, including sclerocytes, rare amoeboid cells, symbiotic bacteria, spicules, as well as gametes and developing embryos during the reproduction season.

The rapid wound healing and high regeneration capacity after different surgical interventions were demonstrated, indicating that *Leucosolenia* are a promising model for sponge regeneration investigations [20]. *Leucosolenia complicata* and *L. variabilis* have been successfully used for different experiments concerning the study of restoration morphogenesis [13, 19, 21–26].

In a recent publication, we revisited various regenerative processes of *L. variabilis* from the White Sea using electron microscopy, laser confocal microscopy, epifluorescence microscopy, and time-lapse microscopy. These approaches allowed us to precisely address the issues of morphogenetic mechanisms, cell transdifferentiations, movements, and proliferation. Our study reveals the contributions of cell types to reparative regeneration in this species and demonstrates a central role of epithelial morphogenesis and transdifferentiations in the regeneration process [13].

*Leucosolenia* demonstrate high and diverse regenerative capacity after various surgical operations. In this chapter, we provide methods to study the reparative regeneration of the body wall, whole-body regeneration (WBR) from amputated tubes and small fragments of the body wall, as well as cell reaggregation and primmorph formation after tissue dissociation. We also provide protocols for cell proliferation, apoptosis, and immunohistochemical studies. Finally, we present approaches for functional analysis of cell proliferation and skeleton synthesis during regeneration.

## 2 Materials

1. Castroviejo scissors.
2. 50 μm nylon mesh.

**Fig. 2** (continued) membrane (24 hpo). (**e**) Cell proliferation is neither affected nor contributes to the regeneration at any stage of the process, for example, during transformation of regenerative membrane into intact body wall (36 hpo), white dashed line delimits regenerative membrane, orange arrowheads mark cells in S phase of cell cycle (EdU-positive cells), white arrowheads—cells in late $G_2$/M-phase of cell cycle (pH3-positive cells). (**f**) Apoptosis during early stages (3 hpo) of regeneration, white dashed line marks wound surface, white arrowheads mark apoptotic (TUNEL-positive) cells. (**g**, **h**) Spicule secretion in the regenerative membrane during its transformation into intact body wall (72 hpo), (**g**) general view under bright field microscopy, (**h**) epifluorescence view with FITC filter set with newly synthesized spicules showing bright green emission, white arrowheads marks the same spicules in (**g**) and (**h**). (**a**, **b**, **c**, **d**, **g**, **h**) WBR from an amputated oscular tube; (**e**) WBR from an amputated cormus tube; (**f**) body wall regeneration. Scale bars: (**a**) 200 μm, (**b**, **e**, **f**, **g**, **h**) 50 μm, (**c**, **d**) 5 μm. *ch* choanocyte, *en* endopinacocyte of regenerative membrane, *ex* exopinacocyte of intact tissue, *f* flagellum, *m* mesohyl, *mv* microvilli of choanocyte, *rm* regenerative membrane, *tch* transdifferentiated choanocyte, *wo* wound orifice

3. Hemocytometer.
4. Orbital shaker.
5. Sterile filtered seawater (FSW): filter fresh seawater through sterile 0.22 μm syringe filter with PES membrane.
6. Laboratory aquarium.
7. Stereomicroscope.
8. Inverted microscope with phase-contrast or DIC objectives.
9. Digital camera compatible with microscopes.
10. Thermo-controlling plate.
11. Glass-bottom Petri dishes.
12. Clearing agent like xylene or similar (e.g., Neo-Clear, Sigma-Aldrich) (*see* **Note 1**).
13. Paraffin waxes or similar (e.g., Paraplast, Sigma-Aldrich).
14. Bouin fixative: 15 mL saturated solution of picric acid, 5 mL 35–40% formalin, 1 mL 100% acetic acid. Store at RT.
15. 0.1 M Millonig phosphate buffer: 13 mM $NaH_2PO_4$, 87 mM $Na_2HPO_4$, 85.55 mM NaCl in distilled water, adjust pH to 7.4–7.5 with 0.1 M HCl. Store at 4 °C, shelf life—up to 1 month.
16. 0.1 M Na-Cacodylate buffer: 0.1 M Na-cacodylate, 85.55 mM NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$ in distilled water, adjust pH to 7.0–7.5 with 0.1 M HCl. Store at RT, shelf life—virtually unlimited.
17. 10% (w/v) ruthenium red aquiferous solution: 0.1 g dry ruthenium red, 1 mL distilled water. Heat solution up to 60 °C for 20 min and cool to RT, centrifuge at max speed and accurately transfer supernatant into a new vessel. Store at 4 °C, shelf life—up to several months.
18. 2.5% (v/v) glutaraldehyde: 1 mL 25% commercially available glutaraldehyde aquiferous solution, 9 mL either buffer. Use freshly prepared or store at 4 °C, shelf life—up to 1 month (*see* **Note 2**).
19. 2.5% (v/v) glutaraldehyde +0.1% (w/v) ruthenium red in Na-Cacodylate buffer: 1 mL 25% commercially available glutaraldehyde aquiferous solution, 0.1 mL 10% (w/v) ruthenium red aquiferous solution, 8.9 mL 0.1 M Na-Cacodylate buffer. Use freshly prepared or store at 4 °C, shelf life—up to 1 month.
20. 1% (v/v) $OsO_4$: 1 mL 4% commercially available $OsO_4$ aquiferous solution, 3 mL either buffer. Use freshly prepared (*see* **Note 2**).
21. 1% (v/v) $OsO_4$ + 0.1% (w/v) ruthenium red in Na-Cacodylate buffer: 1 mL 4% commercially available $OsO_4$ aquiferous

solution, 0.04 mL 10% (w/v) ruthenium red aquiferous solution, 2.96 mL 0.1 M Na-Cacodylate buffer. Use freshly prepared.

22. 5% (w/v) ethylenediaminetetraacetic acid (EDTA) aquiferous solution: 5 g dry EDTA, 50 mL distilled water. Constantly mix with a magnetic stirrer and add 10 M NaOH drop by drop until EDTA completely dissolve, adjust pH to 7.5–8.0 with 1 M and 0.1 M NaOH, fill up to 100 mL with distilled water. Store at RT, shelf life—virtually unlimited.
23. Dehydration Mix 1 (DM1): 3 mL 96% ethanol, 1 mL acetone.
24. Dehydration Mix 2 (DM2): 2 mL 96% ethanol, 2 mL acetone.
25. Dehydration Mix 3 (DM3): 1 mL 96% ethanol, 3 mL acetone.
26. Infiltration Mix 1 (IM1): 2 mL acetone, 1 mL resin.
27. Infiltration Mix 2 (IM2): 2 mL acetone, 2 mL resin.
28. Infiltration Mix 3 (IM3): 1 mL acetone, 2 mL resin.
29. Toluidine blue—methylene blue mixture: 0.5 g sodium tetraborate, 0.5 g toluidine blue, 0.1 g methylene blue in 50 mL distilled water. Mix first the sodium tetraborate with a magnetic stirrer until sodium tetraborate completely dissolve, then add the toluidine blue and methylene blue. Mix with a magnetic stirrer until complete dissolution. Store in the dark at RT, shelf life—virtually unlimited.
30. 4% (w/v) uranyl acetate: 2 g uranyl acetate, 50 mL distilled water. Vigorously mix until completely dissolution. Store in the dark at 4 °C, shelf life—several months.
31. 0.4% (w/v) lead citrate: 0.2 g lead citrate, 50 mL distilled water, 0.5 mL 10 N NaOH. Vigorously mix until completely dissolution. Store in the dark at 4 °C, shelf life—several months.
32. 10× phosphate buffered saline (10× PBS): 1370 mM NaCl, 27 mM KCl, 100 mM $Na_2HPO_4$, 18 mM $KH_2PO_4$ in distilled water, adjust pH to 7.4–7.5 with 0.1 M HCl. Store at RT, shelf life—virtually unlimited.
33. 1× phosphate buffered saline (1× PBS): 10% (v/v) 10× PBS in distilled water. Store at 4 °C, shelf life—up to 1 month.
34. 16% (w/v) paraformaldehyde (PFA) aquiferous solution: 16 g PFA powder, 50 mL distilled water. Add several drops of 1 M NaOH, heat to 60 °C and mix on a magnetic stirrer until complete dissolution of the powder. Adjust pH to 7.4–7.5 with NaOH, fill up to 100 mL with distilled water. Store at 4 °C, shelf life—up to several months.
35. 4% PFA PBS: 1 mL 16% PFA aquiferous solution, 0.4 mL 10× PBS, 2.6 mL distilled water. Use freshly prepared or store at 4 °C, shelf life—up to 1 month (*see* **Note 2**).

36. 0.5% (v/v) Triton X-100 in PBS (PBSt): 0.5 mL Triton X-100, 99.5 mL 1× PBS. Store at 4 °C, shelf life—up to several months.
37. 1% (w/v) BSA PBS: 1 g BSA, 50 mL distilled water. Mix on a magnetic stirrer until complete dissolution, add 10 mL 10× PBS, fill up to 100 mL with distilled water. Filter solution through sterile 0.22-μm syringe filter with PES membrane into a sterile vessel. Store at 4 °C, shelf life—up to 1 month.
38. Blocking solution (BS): 1 g BSA, 0.1 g gelatin from cold water fish skin, 50 mL distilled water. Mix on a magnetic stirrer until complete dissolution of powders, add 0.5 mL Triton X-100, 0.05 mL Tween 20, 10 mL 10× PBS, fill up to 100 mL with distilled water. Filter solution through sterile 0.22-μm syringe filter with PES membrane into a sterile vessel. Store at 4 °C, shelf life—up to 1 month.
39. 30% (v/v) glycerol-PBS/DABCO: 0.25 g DABCO, 7 mL 1× PBS. Mix solution until complete dissolution, add 3 mL glycerol.
40. 60% (v/v) glycerol-PBS/DABCO: 0.25 g DABCO, 4 mL 1× PBS. Mix solution until complete dissolution, add 6 mL glycerol.
41. 90% (v/v) glycerol-PBS/DABCO: 0.25 g DABCO, 1 mL 1× PBS. Mix solution until complete dissolution, add 9 mL glycerol.
42. Anti-phospho-Histone H3 ($pSer^{10}$) primary antibodies produced in rabbit (e.g., H0412, Sigma-Aldrich).
43. Anti-acetylated-α-tubulin primary antibodies produced in mouse (e.g., T6793, Sigma-Aldrich).
44. Fluorescent conjugated anti-mouse (e.g., A21202, Thermo Fisher Scientific) and anti-rabbit (e.g., A-31573, Thermo Fisher Scientific) secondary antibodies.
45. 10 mM EdU stock solution: 50 mg EdU, 19.83 mL DMSO (cell culture grade). Aliquot solution in 500 μL and store at −20 °C, shelf life—up to several years.
46. 0.1 M $CuSO_4$ stock solution: 249.7 mg $CuSO_4 \bullet 5H_2O$, 10 mL distilled water. Store at 4 °C, shelf life—virtually unlimited.
47. 200 mg/mL sodium L-ascorbate stock solution: 2 g sodium L-ascorbate, 10 mL distilled water. Aliquot solution in 200 μL and store it at −20 °C, shelf life—up to several months.
48. 1 mM Sulfo-Cyanine3 Azide stock solution: 1 mg Sulfo-Cyanine3 azide, 1.357 mL distilled water. Aliquot solution in 10 μL and store it at −20 °C, shelf life—virtually unlimited.

49. Click-reaction cocktail: 850 μL 1× PBS, 40 μL 0.1 M $CuSO_4$, 10 μL 1 mM Sulfo-Cyanine3 azide, 100 μL 200 mg/mL sodium L-ascorbate (*see* **Note 3**).
50. TUNEL Imaging Kit (e.g., Click-iT TUNEL Imaging assay, Thermo Fisher Scientific, or In Situ Cell Death Detection Kit, Merck).
51. DNase reaction buffer: 0.1 M Tris–HCl, 25 mM $MgCl_2$, 1 mM $CaCl_2$ in Milli-Q grade water, adjust pH to 7.4–7.5 with 0.1 M HCl. Aliquot buffer in 1 mL and store at −20 °C, shelf life—up to several years.
52. 1 mg/mL aphidicolin stock solution: 1 mg dry aphidicolin, 1 mL DMSO. Aliquot solution in 10 μL and store at −20 °C, shelf life—up to several years.
53. 0.5 M hydroxyurea stock solution: 380 mg dry hydroxyurea, 10 mL distilled water. Aliquot solution in 100 μL and store at −20 °C, shelf life—up to several years.
54. 1 g/L Calcein disodium salt solution: 100 mg dry Calcein, 100 mL distilled water. Store at 4 °C, shelf life—up to several months.

## 3 Methods

### 3.1 Collection, Manipulation, and Laboratory Maintenance of Sponges

1. Collect sponges in the upper subtidal zone (0–2 m) at low tide with their brown algal substrate (*Ascophyllum, Fucus*).
2. During collection, pay special attention to avoid contact of the sponges with air.
3. Maintain collected sponges in a 100 L laboratory aquarium with natural seawater (daily change of half-volume) and biological filters at a temperature of 6–12 °C, not longer than 4 days (*see* **Note 4**).
4. Before surgery, clean the sponge thoroughly of detritus, epibionts, and fouling, using tweezers, soft brush, and pipetting, washed several times with a large volume of FSW.

### 3.2 Surgical Operations and Subsequent Cultivation

The surgical operations are carried out manually under a stereomicroscope, while a sponge is carefully supported with forceps. Four types of surgical operations were performed (Fig. 1b).

#### 3.2.1 Excision of a Small Part

Approximately 0.3–0.5 × 0.3–0.5 $cm^2$ of the body wall is removed (*see* **Note 5**). It could be made in different parts of sponge: at the oscular tube, tubes of cormus or diverticula (Fig. 1b).

1. Make two oblique incisions using Castroviejo scissors to excise a square piece of the sponge body wall.

2. Remove the cut fragment from the Petri dish.
3. Maintain operated sponge in the Petri dish with FSW at 10–12 °C.
4. Change half of FSW with fresh one every 12 h.
5. Inspect and photograph the sponge using a stereomicroscope equipped with a digital camera to monitor regeneration processes. Observations should be done at 3, 6, 12, 18, 24, 36, 48 h postoperation (hpo), and then every 24 h.

#### 3.2.2 Amputation of Oscular Tubes

Removal of an oscular tube (Fig. 1b). After this operation, the regeneration of the basal parts in the amputated oscular tubes could be studied.

1. Cut oscular tubes perpendicular to the main axis using scissors.
2. Remove sponge from the Petri dish.
3. Maintain the amputated oscular tubes in the Petri dish with FSW at 10–12 °C.
4. Change half of FSW with fresh one every 24 h.
5. Inspect and photograph the amputated oscular tubes as indicated above.

#### 3.2.3 Whole-Body Regeneration

Excision and cultivation of fragments of the body wall (Fig. 1b 5) will allow for studying the whole-body regeneration (WBR) of the sponge.

1. Cut off numerous square fragments of the sponge body wall by oblique incisions using Castroviejo scissors.
2. Remove sponge from the Petri dish.
3. Maintain the fragments of the body wall in the Petri dish with FSW at 10–12 °C.
4. Change half of FSW with fresh one every 24 h.
5. Inspect and photograph the fragments of the body wall as indicated above.

#### 3.2.4 Cell Reaggregation

This type of operation (Fig. 1b 6) (*see* **Note 6**) will allow for studying the WBR of the sponge:

1. Cut a sponge in small fragments with scalpel and forceps in FSW.
2. Squeeze fragments of sponge tissues through 50 μm nylon mesh into vessels with fresh FSW to obtain cell suspension.
3. Determine the cell concentration in the obtained suspension using a hemocytometer.

4. Dilute the obtained suspension with FSW up to concentrations of $1 \times 10^7$ to $3 \times 10^7$ cells/mL and cultured in 30 mm Petri dishes (5 mL of suspension in each dish).
5. Change half of FSW with fresh one every 48 h.
6. Inspect and photograph the cell aggregates as indicated above at 12, 24, 36, 48 h postdissociation (hpd), and then every 24 h.

### 3.3 Time-Lapse Recordings

Time-lapse recordings represent a useful tool for long-term continuous observations of regeneration processes (*see* **Note 7**).

1. Place a specimen in a Petri dish under a microscope and provide it with appropriate orientation, allowing for observation of a wound site (Fig. 2a).
2. Use a thermo-controlling plate for controlling the temperature of specimens, keeping it around the normal range for sponges.
3. Use a lateral moderate-intensity illumination as the main light source and the recording period of 0.5–5 min for time-lapse recording using a stereomicroscope.
4. Use objectives 20× and 40× allowing for phase-contrast or DIC observations and the recording period of 10–30 s for time-lapse recording using an inverted microscope.
5. Regularly check the recording scene to monitor the regeneration process stage, the orientation of a specimen, and a focal plane of a microscope.

### 3.4 Methods of Tissue Fixation and Processing for Histological Investigation

1. Fix in Bouin fixative for 2 h at room temperature (RT). Fixative volume should be at least tenfold higher than that of tissues.
2. Rinse specimens with 70% ethanol several times at RT until the yellow color disappear (*see* **Note 8**).
3. Dehydrate specimens in ethanol series (70–70–96–96%) for 15 min each stage at RT.
4. Dehydrate specimens three times in absolute ethanol 20 min each time at RT.
5. Incubate specimen twice in clearing agent for 1 h each time at RT.
6. Infiltrate specimens with the first wax for 1 h at 58 °C.
7. Infiltrate specimens with the second wax for 30 min at 58 °C.
8. Embed specimens in a fresh portion of wax in molds at RT.

### 3.5 Methods of Tissue Fixation and Processing for Transmission Electron Microscopy (TEM)

Two methods of fixations are possible: (a) for conventional observations and (b) for extracellular matrix (ECM) and cell junction studies (*see* **Note 9**). For conventional observation (Fig. 2b, c), the fixation and subsequent treatments can be done using either 0.1 M Millonig phosphate buffer or 0.1 M Na-Cacodylate buffer. Both

buffers provide equal quality of fixations. However, the same one should be used during fixation and treatments of a single specimen. Unless specified, all incubations and rinses are performed at RT "with shaking," that is, with constant orbital shaking at 70 rpm.

1. Fix tissues in 2.5% glutaraldehyde overnight at +4 °C without shaking.
2. Rinse specimens three times with the selected buffer, for 30 min each time.
3. Postfix specimens in 1% $OsO_4$ for 2 h at RT in the dark.
4. Rinse specimens three times with the selected buffer for 30 min each time.
5. Quickly rinse specimens with distilled water at RT.
6. Incubate specimens in 5% EDTA solution for calcareous spicule dissolution for 2 h (*see* **Note 10**).
7. Quickly rinse specimens with distilled water at RT.
8. Rinse specimens three times with the selected buffer for 30 min each time.

The fixation and subsequent treatments for ECM and cell junction visualization should be done using 0.1 M Na-Cacodylate buffer, as phosphate ions block ruthenium red interactions with tissues.

1. Fix tissues in 2.5% glutaraldehyde + 0.1% ruthenium red for 2 h at +4 °C without shaking (*see* **Note 11**).
2. Rinse specimens three times with the Na-cacodylate buffer for 30 min each time.
3. Postfix specimens in 1% $OsO_4$ + 0.1% ruthenium red for 3 h at RT (*see* **Note 11**).
4. Rinse specimens three times with the Na-cacodylate buffer for 30 min each time.
5. Quickly rinse specimens with distilled water at RT.
6. Incubate specimens in 5% EDTA solution for spicule dissolution for 2 h.
7. Quickly rinse specimens with distilled water at RT.
8. Rinse specimens three times with Na-cacodylate buffer for 30 min each time.

After fixation, specimens should be dehydrated and embedded "with shaking" until **step 14**:

9. Dehydrate specimens in ethanol series (10–20–30–40–50–60–70–70–82–96–96%) 15 min each stage at +4 °C.
10. Dehydrate specimens in dehydration mixes (DM1-DM2-DM3) 20 min each stage at +4 °C.

11. Dehydrate specimens twice in acetone 20 min each time.
12. Infiltrate specimens in infiltration mixes (IM1-IM2-IM3) 2–12 h each stage at +4 °C in tightly sealed containers.
13. Infiltrate specimens with resin in open containers for 6 h at RT.
14. Infiltrate specimens with fresh resin for additional 4 h at RT.
15. Fill a flat embedding mold with fresh resin without shaking.
16. Transfer specimens in the flat embedding mold and orient them properly.
17. Polymerize resin according to the manufacturer's instructions (typically 1–2 days at +60 °C).
18. Trim a resin block with a razor blade to remove excessive resin around the specimen and form a truncated pyramid.
19. Cut semithin sections 0.5–1 μm thick using an ultramicrotome (e.g., Leica EM UC6) and transfer then onto a clean object slide.
20. Stain semithin sections with toluidine blue—methylene blue mixture 30–90 s at +60 °C using a hotplate.
21. Wash stained sections twice with distilled water, dry them and make permanent preparation using the resin as a mounting medium.
22. Polymerize the resin in the permanent preparations for 1–2 days at +60 °C.
23. Study the preparations with a brightfield microscope (e.g., Leica DM2500) and localize the region of interest for TEM.
24. Cut ultrathin sections of the region of interest from the same resin block using an ultramicrotome and transfer them onto grids for electron microscopy.
25. Stain grids with 4% aqueous uranyl acetate for 30–60 min at +37 °C (*see* **Note 12**).
26. Carefully wash grids three times with distilled water and dry them.
27. Stain grids with 0.4% lead citrate for 15–30 min in the dark and in the presence of granulated NaOH at RT.
28. Carefully wash grids three times with distilled water and dry them.
29. Study grids with a transmission electron microscope.

The fixation, postfixation, and dehydration of specimens should be done as detailed in **steps 1–11** in Subheading 3.5 (*see* **Note 13**).

### 3.6 Methods of Tissue Fixation and Processing for Scanning Electron Microscopy (SEM)

1. Dry specimens in $CO_2$ critical point using critical point drier (e.g., Hitachi HCP-2).
2. Mount samples on metal specimen mounts or stabs with carbon adhesive glue, carbon adhesive tabs, or nail polish.
3. Metalize mounted specimens using ion coater (e.g., Eiko IB-3).
4. Study specimens using a scanning electron microscope (Fig. 2d).

### 3.7 Cell Proliferation and Immunohistochemical Studies

For cell proliferation studies, a combination of 5-ethynyl-2-′-deoxyuridine (EdU), labeling cells in S-phase of the cell cycle (DNA-synthesizing cells), and anti-phospho-histone H3 antibodies, labeling cells in late$G_2$/M-phase of the cell cycle (dividing cells), is used (Fig. 2e). Unless specified, all incubations and rinses are performed at RT "with shaking," that is, with constant orbital shaking at 70 rpm.

1. Incubate alive regenerating specimens in a 30 mm plastic Petri dish with 5 mL of 20 μM EdU solution in FSW for 6 h at 10–14 °C without shaking (*see* **Notes 14** and **15**).
2. Rinse the specimens twice with fresh FSW, 10–15 min each time, at 10–14 °C.
3. Fix specimens with 4% PFA PBS for 2–12 h at 4 °C without shaking (*see* **Note 16**).
4. Rinse fixed specimens three times with 1× PBS, 30 min each time.
5. Incubate specimens in 5% EDTA solution for calcareous spicule dissolution for 2 h (*see* **Note 10**).
6. Rinse fixed specimens three times with 1× PBS, 30 min each time.
7. Incubate specimens twice in 1% BSA PBS, 20 min each time.
8. Permeabilize specimen with PBSt for 10–15 min.
9. Incubate specimens twice in 1% BSA PBS, 20 min each time.
10. Incubate specimens in freshly prepared Click-rection cocktail for 30–60 min in the dark.
11. Rinse specimens three times with BS, 1 h each time, in the dark.
12. Incubate specimens in the mix of primary antibodies, anti-phospho-histone H3 (rabbit; 1:500–1:1000 dilution) + anti-acetylated-α-tubulin (mouse; 1:1000–1:2000 dilution), in BS overnight in the dark at 4 °C (*see* **Notes 17** and **18**).
13. Rinse specimens twice with BS, 1 h each time, in the dark.
14. Incubate specimen in fresh BS overnight in the dark at 4 °C.

15. Incubate specimens in the mix of secondary antibodies, donkey anti-mouse IgG Alexa Fluor 488 (1:2000 dilution) + donkey anti-rabbit IgG Alexa Fluor 647 (1:2000 dilution), in BS overnight in the dark at 4 °C.
16. Rinse specimens twice with 1× PBS, 1 h each time, in the dark.
17. Incubate specimen in fresh 1× PBS overnight in the dark at 4 °C.
18. Counterstain nuclei with 2 μg/mL DAPI solution in 1× PBS for 1 h in the dark.
19. Rinse specimens three times with 1× PBS, 30 min each time, in the dark.
20. Infiltrate specimens in 30%–60%–90% glycerol-PBS/DABCO series, 30–60 min at each step (until specimens completely submerge into each solution) in the dark at RT without shaking.
21. Mount specimens in fresh 90% glycerol-PBS/DABCO, using small plasticine spacers in-between object and cover slide to avoid severe deformations of specimens.
22. Seal preparation with nail polish.
23. Study preparations with a confocal microscope using following (or close) excitation wavelengths: 405 nm (DAPI), 488 nm (acetylated-α-tubulin), 555 nm (EdU), 647 nm (phosphohistone H3) (*see* **Note 19**).

### 3.8 Cell Proliferation Blocking

1. Incubate sponge tissue in FSW with 1 μg/mL for aphidicolin or 5 mM for HU for at least 12 h at 10–12 °C to completely block cell proliferation (*see* **Notes 14** and **20**).
2. Change media every 12–24 h, maintaining a constant concentration of a blocking agent, if prolonged blocking is required.
3. Wash sponge tissues in a large volume of fresh FSW and incubate it for at least 24 h at 10–12 °C to release cell proliferation.

### 3.9 Apoptosis Studies

The following protocol is a slightly modified manufacturer's protocol for Click-iT TUNEL Imaging assay (Thermo Fisher Scientific) and In Situ Cell Death Detection Kit (Merck) (*see* **Notes 21** and **22**). Unless specified, all incubations and rinses are performed at RT "with shaking," that is, with constant orbital shaking at 70 rpm.

1. Fix specimens with 4% PFA PBS for 2–12 h at 4 °C without shaking (*see* **Note 16**).
2. Rinse fixed specimens three times with 1× PBS, 30 min each time.
3. Incubate specimens in 5% EDTA solution for calcareous spicule dissolution for 2 h (*see* **Note 10**).

4. Rinse fixed specimens three times with 1× PBS, 30 min each time.
5. Permeabilize specimens with PBSt for 20 min.
6. Rinse fixed specimens three times with 1× PBS, 10 min each time.
7. Incubate specimens in TdT buffer for 30 min.
8. Incubate specimens in TdT-cocktail for 8–12 h (*see* **Notes 23** and **24**).
9. Skip **steps 10** and **11** if not using the Click-iT TUNEL Imaging assay (Thermo Fisher Scientific).
10. Incubate specimens twice in 3% BSA PBS, 10 min each time.
11. Incubate specimens in freshly prepared Click-reaction cocktail for 30–60 min in the dark (*see* **Note 24**).
12. Rinse fixed specimens three times with 1× PBS, 30 min each time, in the dark.
13. Counterstain nuclei with 2 μg/mL DAPI solution in 1× PBS for 1 h in the dark.
14. Rinse specimens three times with 1× PBS, 30 min each time, in the dark.
15. Infiltrate specimens in 30%–60%–90% glycerol-PBS/DABCO sequence, 30–60 min at each step (until specimens completely submerge into each solution) in the dark at RT without shaking.
16. Mount specimens in fresh 90% glycerol-PBS/DABCO, using small plasticine spacers in-between object and cover slide to avoid severe deformations of specimens.
17. Seal preparation with nail polish.
18. Study preparations with a confocal microscope using 405 nm excitation wavelengths for DAPI and excitation wavelength recommended in the manufacturer's protocol for apoptotic cells (Fig. 2f) (*see* **Note 19**).

### 3.10 Skeleton Synthesis Studies

As spicules of *Leucosolenia* are composed of calcium carbonate ($CaCO_3$), their synthesis could be visualized in vivo, using Calcein disodium salt solution (*see* **Note 25**).

1. Incubate regenerating specimen in 100 mg/L Calcein disodium salt solution in FSW at 10–12 °C (*see* **Note 14**).
2. Rinse specimen twice with FSW, 10 min each time at 10–12 °C.
3. Study specimen in an epifluorescent microscope, using a standard FITC filter set (Fig. 2g, h) (*see* **Note 25**).

## 4 Notes

1. Many reagents used in the protocols are health hazardous and requires safety precautions. All fixative solutions (glutaraldehyde, $OsO_4$, PFA, Bouin fixative) should be manipulated with laboratory gloves to avoid damaging skin and in a fume hood, as they are corrosive to the respiratory tract. Similar precautions should be kept during work with acids and alkali. Uranyl acetate is a radioactive substance. DAPI and EdU incorporate in DNA and aphidicolin and HU interferes with DNA synthesis, thus these reagents could potentially have carcinogenic effects.
2. Properly prepared solutions are the first essential step in any protocol. Storage conditions and the state of solutions are no less important. Always check state of solutions before applying them to specimens: all solutions should be clean, without any precipitate or other foreign particles. While the majority of solutions could be preliminary prepared and stored in the appropriate conditions, some of them are recommended to use freshly prepared. We highly recommend using all aldehyde-based fixatives (2.5% glutaraldehyde and 4% PFA PBS) freshly prepared. $OsO_4$-based fixatives must be used only freshly prepared, as $OsO_4$ is quickly reduced in solution, becoming ineffective for tissue fixation.
3. The way of the preparation of the Click-cocktail for Click-reaction is essential. This solution should be freshly prepared for each treatment, and the reagents should be added into 1× PBS in the strict order: $CuSO_4$, Sulfo-Cyanine3 Azide, sodium L-ascorbate; otherwise, the reaction will not proceed. After the addition of $CuSO_4$, some precipitate can appear; this precipitate should dissolve after the addition of sodium L-ascorbate. The stock solution of sodium L-ascorbate should be light-ochre. If the solution changes color to dark-ochre or brown during storage, it should be discarded.
4. A successful experimental study of any organism in a laboratory usually requires adaptation of widely known protocols. Firstly, the conditions during maintaining and experimental manipulation with an organism should be similar to the natural conditions in which the organism lives. In the case of *L. variabilis* from the White Sea, we use natural seawater and maintain physiological for the sponge range of temperatures (6–12 °C). Secondly, all solutions for fixations and treatments should be adopted to the water salinity normal to the studied organism. Here we use solutions adapted to the salinity of 25–26‰ (~750–800 mOsm).
5. The reparative regeneration of the body wall is the best-studied regeneration process in *Leucosolenia* (Fig. 1b1). This type of

regeneration occurs after the excision of a small part of the body wall or amputation of an oscular tube. The process ends within 4–6 days post-operation (hpo) with complete restoration of the lost body wall. Three main stages could be distinguished in this type of regeneration: (1) internal milieu isolation (3—12 hpo), (2) wound orifice healing (regenerative membrane formation) (12—24 hpo) (Fig. 2a), and (3) transformation of the regenerative membrane into an intact body wall (48—144 hpo) [13].

6. Every cell suspension for cell reaggregation experiments should be obtained from only one individual to avoid undesired allorecognition effects. The optimal cell concentrations in obtained suspensions are $1 \times 10^7$ to $3 \times 10^7$ cells/mL. The cell reaggregation will be slowed or even impossible with lower concentrations, while higher concentrations will lead to the formation of single large cell aggregate with low viability [27, 28]. We also recommend maintaining cell cultures on an orbital shaker (70 rpm) for the first 24 h for intensification the cell reaggregation process.
7. Time-lapse recordings represent a powerful instrument for directly assessing morphogenetic events and cellular behavior in a living specimen. Depending on the level at which a regeneration process should be observed, time-lapse recordings could be done using either a stereomicroscope or inverted microscope equipped with a digital camera. Time-lapse recording made with stereomicroscope allows for making a general description of the regeneration process and some morphogenetic events, while recordings with an inverted microscope allow for observing the behavior of single cells. The maintaining of appropriate conditions for a living specimen during long-term time-lapse recordings is of paramount importance. For constant temperature management in microscope systems, thermo-controlling plates represent the best solution. However, a microscope for time-lapse recordings could be placed directly to thermo-stable conditions: into a fridge (for smaller microscope models) or cold-room (for large microscope models). Additionally, the medium in which a specimen is maintained should be periodically changed with fresh one.

   The illumination is no less important. Not all light sources are equally suitable to observe living specimens, especially long term during time-lapse recordings. Avoid using heating light sources, as they will quickly raise the temperature of a specimen. Also, pay attention to the wavelength profile of a light source, as a light source with high intensities in the “blue” part of the spectrum may have deleterious effects on a living specimen. For time-lapse recording with a stereomicroscope, a lateral illumination gives a better contrast to the specimens. It also

could be combined with transmission illumination. The light intensity should be set to a moderate level, as too bright illumination could negatively affect the viability of a specimen. The optimal recording period depends on the studied process: the quicker process—the shorter period should be used. Usually, a set of preliminary recordings are required to determine the optimal period.

8. Complete rinse of specimens after Bouin fixative with 70% ethanol could take up to several days.
9. For the best fixation quality for transmission electron microscopy, specimens should be treated and embedded in resin as soon as possible. However, if necessary, specimens can be stored in the buffer after postfixation or spicule dissolution for weeks and in 96% ethanol after dehydration for days. The storage temperature should be +4 °C.
10. Spicule dissolution with 5% EDTA solution is appropriate only for calcium carbonate spicules. If you study demosponges, hexactinellids, or homoscleromorphs with silica spicules, use 4–10% HF solution to dissolve spicules. If you study a sponge without an inorganic skeleton, omit step with spicule dissolution.
11. Pay special attention to the fixation timing in the protocol of ECM and cell junction fixation for electron microscopy, as it is essential to successful results (especially postfixation in buffered 1% $OsO_4$ + 0.1% ruthenium red).
12. During the staining of ultrathin sections, special attention should be paid to avoid the appearance of dust on the sections. We recommend working in a laboratory with minimal air movement. Wash working space before the start of the staining, and filter all used solutions (including distilled water) with 0.22 μm syringe filters with PES membrane. The uranyl acetate is a radioactive substance and requires special precautions during working with. Otherwise, it could be substituted with nonradioactive analogs (e.g., UranyLess EMS). Special attention should be paid to minimize contact of lead citrate solution (especially during staining) with air. Lead citrate will form an insoluble precipitate upon interaction with air $CO_2$. We recommend staining ultrathin sections with lead citrate in close Petri dish in the presence of granulated NaOH, which will reduce $CO_2$ concentration in the dish. In general, staining with lead citrate could be omitted, but ultrathin sections stained with it will show more contrast.
13. If it is necessary to retain spicules for scanning electron microscopy studies, the **steps 5–8** in Subheading 3.5 should be omitted.

14. After the addition of a reagent into the FSW with a living specimen, mix the solution well by pipetting or short incubation (10–20 min) on an orbital shaker (70 rpm). It is especially important for reagents with stock solutions made in DMSO.
15. Several technical control specimens should be used to control the quality of EdU staining and correctly interpret obtained Z-stacks, discriminating between specific and unspecific signals:
    (a) Negative control specimen (NCS), an alive regenerating specimen, which is incubated in DMSO solution in FSW at **step 1** (instead of EdU solution, as in experimental specimens). Each batch of experimental specimens should be supplemented with NCS. Incubate NCS in a 30 mm plastic Petri dish with 5 mL of FSW supplemented with DMSO for 6 h at 10–14 °C in parallel with the experimental specimens according to Subheading 3.7. The volume of added DMSO is equal to the volume of the EdU stock solution added to the experimental specimens. After incubation, treat NCS similarly to experimental specimens. The staining patterns in EdU-channel (555 nm) in NCS should be recognized as unspecific, and similar patterns in experimental specimens should not be considered.
    (b) Positive control specimen (PCS), any alive specimen, which admittedly contains DNA-synthesizing cells. We recommend supplying each batch of experimental specimens with PCS. Treat PSC similarly and parallel to the experimental specimens, starting from **step 1** of Subheading 3.7. If, during the study with a confocal microscope PCS shows, no EdU-positive cells, then experimental specimens should be discarded as some issues with EdU incubation or Click-reaction arose.
16. Specimens fixed for cell proliferation studies could be stored up to 6 months and for immunohistochemical studies—up to 1–6 months, depending on an antigen of interest. Storage conditions: 4% PFA PBS, 4 °C. Specimens for apoptosis studies should be processed as soon as possible, as prolonged storage could lead to the intensive DNA fragmentation and, subsequently, to the false-positive results of TUNEL assay.
17. The sponge tissues could be studied by immunohistochemical methods, using antibodies. Although no sponge-specific primary antibodies are commercially available, numerous commercial primary antibodies, recognizing epitopes in various vertebrates and invertebrates, work properly in sponge tissues.

Immunohistochemical studies could be done in parallel with EdU cell proliferation studies (in this case, apply primary and secondary antibodies at **steps 10** and **12** in Subheading 3.7, respectively) or separately (in this case, omit **steps 1–2**, **5**, and **7–8** in Subheading 3.7).

At least at several first treatments, two negative control specimens should be used to control the quality of immunohistochemical staining and correctly interpret obtained Z-stacks, discriminating between specific and unspecific signals:

(a) Primary antibodies NCS. Incubate this specimen in BS instead of primary antibodies at **step 10**.

(b) Secondary antibodies NCS. Incubate this specimen in BS instead of secondary antibodies at **step 12**.

(c) The staining patterns in both NCS should be recognized as unspecific, and similar patterns in experimental specimens should not be considered.

18. If several primary antibodies are used in immunohistochemical studies, they should be produced in different host species (mouse, rabbit, chicken, etc.). In turn, secondary antibodies should be chosen according to host specificity of primary antibodies and carry fluorescent dyes with excitation wavelengths not overlapping with each other and Click-reaction azide (in the case of parallel staining with EdU).

19. Preparations of stained specimens for cell proliferation, apoptosis, or immunohistochemical studies could be stored up for 1–2 months in the dark at 4 °C.

20. Functional tests are an essential step required to make conclusions about the involvement of cell proliferation into a regenerative process. Two chemical agents, aphidicolin and hydroxyurea (HU), allow for complete blocking of cell proliferation at S phase of the cell cycle in intact and regenerating tissue of *L. variabilis*. Aphidicolin is a reversible inhibitor of DNA polymerase α and δ, while HU is a reversible inhibitor of ribonucleotide reductase (RNR). The minimal effective concentrations were determined as 1 μg/mL for aphidicolin and 5 mM for HU. Both agents do not show evident negative effects on the general viability of sponges even during prolonged expositions up to 5 days. 0.5 M HU stock solution could be prepared directly in FSW, instead of distilled water.

21. Two commercially available apoptosis detection kits, which use TUNEL assay, are suitable for sponge tissues: Click-iT TUNEL Imaging assay (Thermo Fisher Scientific) and In Situ Cell Death Detection Kit (Merck). Both kits label double-strand DNA breaks generated during the late stages of

apoptosis. In Situ Cell Death Detection Kit is based on one-step labeling through the incorporation of dUTP conjugated with fluorescent dye in cell DNA at double-strand break sites. In turn, Click-iT TUNEL Imaging assay is based on two-step labeling: at the first step, dUTP modified with alkyne (EdUTP) is incorporated in cell DNA at double-strand break sites, at the second—Click-reaction visualizes EdUTP with Alexa Fluor azide. While In Situ Cell Death Detection Kit offers simpler and shorter treatments, Click-iT TUNEL Imaging assay gets an advantage of better penetration in tissue due to the small size of both EdUTP and Alexa Fluor azide.

22. Two technical control specimens should be used to control the quality of TUNEL staining and correctly interpret obtained Z-stacks, discriminating between specific and unspecific signals:
    (a) Negative control specimen (NCS), a specimen, in which the TdT reaction at **step 6** is blocked. Treat NCS similarly to experimental specimens but incubate it TdT-cocktail devoid of TdT-enzyme. The staining patterns in TUNEL-channel in NCS should be recognized as unspecific, and similar patterns in experimental specimens should not be considered.
    (b) DNase positive control specimen (DNase-PCS), a specimen (intact sponge tissues), in which dsDNA breaks are artificially introduced by DNase I. Treat DNase-PCS similarly to experimental specimens but incubate it in DNase I solution containing 1–2 U of the enzyme for 30 min at 37 °C immediately prior to **step 5**. DNase I incubation solution could be prepared by mixing commercially available DNase I enzyme, 10× DNase reaction buffer, and appropriate volume of MilliQ. After DNase treatment, rinse DNase-PCS three times with 1× PBS, 10 min each time, and proceed to **step 5**.
    (c) As DNase I is highly volatile, to avoid contamination of experimental samples with it (which will generate pseudo-positive staining) use a separate set of instruments for manipulations with DNase-PCS. If during the study with a confocal microscope, DNase-PCS shows no TUNEL-positive cells, then experimental specimens should be discarded as some issues with TdT-reaction or Click-reaction arose.
23. TUNEL assay for apoptotic cell detection is based on an enzymatic reaction of TdT. DNase-PC processing also includes an enzymatic reaction of DNase I. The activity of both enzymes highly depends on the storage conditions and could vary from batch to batch. Thus, if you have issues with TUNEL assay, you

should try to change enzymes on fresh ones. Also, you could try to extend the duration of enzymatic reactions and raise reaction temperature to 37 °C.

24. The composition and preparation instructions for TdT- and Click-iT cocktails vary in Click-iT TUNEL Imaging assay (Thermo Fisher Scientific) and In Situ Cell Death Detection Kit (Merck) and should be checked in the manufacturer's protocol for a particular kit. Use only Milli-Q grade water to prepare both cocktails.
25. Only Calcein disodium salt (not Calcein-AM) is appropriate for in vivo studies of spicule synthesis. The stock solution of Calcein disodium salt solution could be prepared directly in FSW, instead of distilled water. Incubation time in Calcein solution could be varied: short incubation time (several hours) is suitable for studies of spicule synthesis rate, prolonged incubation time (1–2 days)—for studies localization of spicule synthesis sites and description of general patterns of skeleton restoration. If needed, after in vivo study, the specimen could be further incubated in the Calcein solution for additional labeling. Alternatively, specimens could be fixed and processed for immunohistochemical studies (*see* Subheading 3.7; omit **step 5** in this protocol to retain spicules in the specimen).

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