Chapter 16

Studying Tunicata WBR Using *Botrylloides anceps*

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Abstract

Tunicates are marine filter-feeding invertebrates that can be found worldwide and which are the closest phylogenetic group to the vertebrates (Craniata). Of particular interest, colonial tunicates are the only known chordates that can undergo Whole-Body Regeneration (WBR) via vascular budding. In *Botrylloides anceps*, a fully functional adult regenerates from a fragment of the vascular system in around 2 weeks after amputation. In this chapter, we present protocols to collect *B. anceps* colonies, confirm their species, breed them in the lab, monitor WBR and perform histological staining on cryosections.

Key words Whole-body regeneration, *Botrylloides anceps*, Vascular budding, DNA barcoding, Chordate, Histological section

1 Introduction

Tunicates are filter-feeding invertebrates that have colonized virtually all marine habitats. Although they were classified in the Mollusca phylum during the early twentieth century, the Tunicata subphylum belong to the Chordata and is the closest phylogenetic group to the vertebrates (Craniata) [1]. Consequently, and despite their apparently simpler body morphologies, tunicates display all chordate features (notochord, post-anal tail, endostyle, neural tube and gill slits) as well as a relatively high tissue complexity (heart, neural ganglion, tunic, circulatory system) [2]. Tunicates is a very diverse group of animals that displays quite different reproductive features, repair abilities, development strategies, and life cycles [3–5]. The majority of tunicates are sessile hermaphrodites that reproduce through a motile tadpole larval stage. After a short free-swimming life stage [2], the tadpole settles on a substrate using the adhesive papillae located at tip of its head. It undergoes a rapid metamorphosis during which its tail and notochord are resorbed, its organs mature and filter-feeding starts. Water enters through the oral siphon, is filtered by the pharyngeal basket and is evacuated through the atrial siphons [6, 7].

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In addition to sexual reproduction, a number of tunicates can reproduce asexually by budding in a process termed blastogenesis (reviewed in [8]). The adult animal, called zooid, starts the development of its daughter, called bud, by the thickening of its epithelium together with that of the underlying layer of tissue. The location of the bud and thus the origin of the underlying tissue can vary depending on the species [8]. These tissue invaginate until forming a double vesicle stage common to all types of asexual reproduction in tunicates. The inner layer will then further invaginate to produce the various organs and the whole bud will mature until it becomes a filter-feeding zooid. In some species of budding tunicates, buds remain connected to its zooid, typically through an interzooidic vascular system, thus forming colonies. In some colonial tunicates, in particular among members of the *Botrylloides* and *Botryllus* sister genera, blastogenesis is a highly synchronized process where the new generation of buds matures simultaneously throughout the colony to replace the old zooids that get resorbed during the so-called takeover stage.

In botryllid tunicates, researchers have identified a second nonembryonic development that can lead to the formation of zooids. Botryllids can undergo whole-body regeneration (WBR) from a fragment of their interzooidic vascular system [4, 9]. Most notably, this is the only know occurrence of WBR in the Chordata phylum. WBR is a type of vascular budding, which is initiate by an injury that leads to the loss of all zooids and buds from the colony [9–11]. Many of the up-regulated metabolic pathways during the WBR play a crucial role in stem cell maintenance, proliferation, differentiation, and tissue organization [12–15]. Pluripotent stem cells (potentially undifferentiated hemoblasts) are assumed to be the precursor cells for WBR [4, 9, 16, 17]. It has been reported that regeneration in *Botrylloides leachii* is initiated by the activation of population of dormant stem-like cells that line the surface of the vascular epithelium [14, 16, 18]. In *Botrylloides diegensis*, a population of *Integrin alpha 6* positive circulating stem cells have been shown to be the source of the WBR capacity [19]. In both cases, upon activation, these cells migrate to the vessel lumen where regeneration begins, and these precursor cells differentiate and eventually transform into a single adult individual within the regeneration lumen [9, 16, 17, 20]. The epithelial layer close to the wound area is generally thought to be the origin of the activation source for regeneration [13, 21].

Vascular budding has also been reported in *Botrylloides gascoi* and *Botrylloides leachii* under field conditions when colonies recover from their aestivation during which all zooids are lost [22, 23]. Interestingly, vascular budding is a part of the life history of *Botryllus primigenus* where it happens spontaneously throughout its adult life cycle [24–26]. Altogether, botryllid ascidians display the rare feature of using three distinct developmental

pathways to produce the same final organism. This property is of particular interest for comparative developmental studies. Moreover, botryllid ascidians are used as model organisms in a wide range of studies including apoptosis, immunobiology, allorecognition and angiogenesis [4, 18, 20, 27–32]. These animals are thus highly suitable as research specimens, their usage will be widely popularized in the near future.

To promote these exciting organisms, we here present a number of protocols for the study of colonial tunicates that we developed for *Botrylloides anceps*. The species originates from the Pacific Ocean. It was recorded for the first time in the Mediterranean Sea along the coastline of Israel in 2009, most likely after an opportunistic migration through the Suez channel [7]. More recently, we logged this species on Turkish coasts in 2018. In this chapter, we present protocols to collect, identify, induce WBR and study the regenerative process using histological staining of cryosections. These protocols should be readily applicable to other botryllids, and likely to other sessile colonial tunicates as well.

2 Materials

2.1 Animals Collection, Handling, Feeding, and DNA Barcoding

All solutions should be prepared with ultrapure water and stored at room temperature unless otherwise state.

1. Filtered seawater (FSW): 20 μm filtered natural seawater.
2. Husbandry system: containers filled with FSW, air pump, air stones, flexible hose.
3. Cotton thread.
4. Salinity meter (e.g., a seawater refractometer).
5. Single-edged razor blades.
6. Large 75 × 50 mm glass microscopy slides.
7. Freeze-dried rotifer powder.
8. Commercial culture of Ochrophyta single-cell algae (e.g., *Nannochloropsis sp.*).
9. Powdered food for filter feeders (e.g., Coral food SPS, Tropical Marine).
10. Food mixture: 13 mL algae, 1 g powdered food, 1 g freeze-dried rotifer powder in 487 mL H_2O, aliquot in 15 mL tubes, store at −20 °C.
11. 1× Phosphate Buffer Solution (PBS): 1.9 mM NaH_2PO_4, 8.4 mM Na_2HPO_4, 175.0 mM NaCl, adjust pH to 7.4 with HCl.
12. Stereomicroscope equipped with a camera.

13. Lysis stock solutions: Prepare separately 1 M Tris–borate (pH 8.2) solution, 0.5 M EDTA solution, 10% (w/v) SDS solution, and 0.5 M NaCl solution. Use within 2 months.
14. Lysis buffer: 12.5 mL 1 M Tris–borate solution, 10 mL 0.5 M EDTA, 10 mL 10% (w/v) SDS, 1 mL 0.5 M NaCl. Adjust the volume to 50 mL with double distillated H_2O.
15. 5 M $NaClO_4$ stock solution. Prepare fresh every time.
16. Chloroform–isoamyl alcohol: 24 mL chloroform, 1 mL isoamyl alcohol.
17. Phenol–chloroform–isoamyl alcohol: 25 mL phenol (saturated pH 7.5–8.0), 24 mL chloroform, 1 mL isoamyl alcohol.
18. Nanodrop spectrophotometer (A230/A260/A280 nm wavelength).
19. 2× PCR master mix: 20 mM Tris–HCI, pH 8.3, 100 mM KCI, 3 mM $MgCI_2$, 0.002% (w/v) gelatin, 0.4 mM dNTP mix (dATP, dGTP, dCTP, dTTP), stabilizers, 0.06 unit/μL Taq DNA polymerase.
20. Cytochrome oxidase I primer mix: 10 pmol/μL Forward primer 5-AMWAATCATAAAGATATTRGWAC-3, 10 pmol/μL Reverse primer 5- AARAARGAMGTRTTRAAATTHC GATC-3 (*see* **Note 1**).
21. Tris/Borate/EDTA (TBE) buffer: 89 mM Tris–borate, 2 mM EDTA, pH 8.3.
22. Agarose gel: 1.25 g agarose in 100 mL TBE buffer. Heat until the solution is completely clear, add 5 μL of nucleic acid staining solution, gently mix the solution. Pour into a gel tray with comb, cool until solidified.
23. 6× agarose loading dye: 10 mM Tris–HCl, pH 7.6, 0.15% (w/v) orange G, 0.03% (v/v) xylene cyanol FF, 60% (v/v) glycerol, 60 mM EDTA.
24. PCR purification kit.
25. Sanger sequencing service (e.g., supplied from a commercial sequencing company).

2.2 Histological Cryosectioning and Staining

1. Cryostat microtome sectioning machine.
2. Crystal menthol.
3. Bouin's fixative: 75 mL saturated picric acid, 25 mL formalin, 5 mL glacial acetic acid (*see* **Note 2**).
4. Dehydration solution: 30% (w/v) sucrose.
5. Optimal cutting temperature (OCT) compound (e.g., Polar-Stat Plus, Ted Pella).
6. Embedding molds (e.g., Peel-a-way, Polysciences).
7. Coated microscopy glass slides (e.g., Superfrost Plus, VWR).

8. Groat's hematoxylin: 1 g ammonium iron (III) sulfate in 50 mL of H_2O, 0.8 g sulfuric acid mixed with 0.5 g hematoxylin in 50 mL EtOH, mix both solutions, filter. Stain can be reused virtually endlessly if filtered every 20–30 stains.
9. Differentiation solution: 0.1% (v/v) HCl in 70% (v/v) EtOH. Can be used to differentiate up to 30 slides.
10. Eosin: 0.1 g Eosin Y, 0.5 mL glacial acetic acid in 100 mL H_2O. Filter, store in a light-protected container.
11. 80% (v/v) EtOH: 20 mL H_2O in 80 mL EtOH.
12. Histological clearing agent (e.g., HistoClear II, National Diagnostics).
13. Mounting medium.

3 Methods

3.1 Sampling and Adaptation

Botrylloides anceps colonies can be found in stony areas of the intertidal zone, less than 1 m deep. So far, we collected this species from three different stations (Konacık-Iskenderun, Mezitli-Mersin, and Alanya-Antalya) but we have found other suitable colonial ascidians in different sites of the North-eastern Mediterranean coastlines (Fig. 1a, *see* **Note 3**).

1. Chose a rocky shore that *B. anceps* possibly inhabits (Fig. 1b, *see* **Note 4**).
2. Sample the selected shore at low tide.
3. Record the salinity of the water (*see* **Note 5**).
4. Record the water temperature.
5. Carefully lift submerged stones to check for colonies on its substrate-facing side.
6. Place back empty stones in the exact same place where collected.
7. Place a marker on the spot of a suitably inhabited rocks (*see* **Note 6**).
8. Carry the stone to the shore for easier manipulations.
9. Give a unique identification code to the colony.
10. Write the code on a glass slide and on a 1.5 mL tube using a waterproof pen.
11. Place the slide and tube nearby to the colony.
12. Take a picture of the whole setup (Fig. 1c).
13. Peel a 1 mm stretch of the colony from its substrate using single-edged razor blades.
14. Place the animals onto the labeled slide (*see* **Note 7**).

Fig. 1 Collecting wild botryllids. (**a**) The map shows the sampling locations from the southern part of Turkey. *Botrylloides* were observed in all four location, but *B. anceps* could not be found in Kızkalesi-Mersin. (**b**) A picture from the Kızkalesi station. (**c**) A picture of a complete setup with an inhabited rock, a fragment transferred on a glass slide and a tube with a sample for DNA barcoding. (**d**) Attaching a sample on a slide. (**e**) Magnification of a *B. anceps* colony secured on a slide by using fine cotton thread

15. Gently tie the colony to the slide using a cotton thread (Fig. 1e, *see* **Note 8**).
16. Put the slide vertically in the fitted slots of the glass staining racks placed inside an insulated plastic container filled up with seawater (*see* **Note 9**).

17. Peel another 1 mm stretch of the colony.
18. Place the sample in the labeled tube filled with 70% ethanol. Keep at room temperature until DNA isolation for DNA barcoding (*see* **Note 10**).
19. Bring back the stone to that exact same place where it was collected from.
20. Transfer the animals and the samples to an aquaculture room within the next 24 h (*see* **Note 11**).
21. Fill the husbandry system with FSW of the same salinity as at the collection place.
22. Put the slides with their staining racks into the husbandry system (Fig. 2a, b, *see* **Note 12**).
23. Aerate the system with a medium bubbling level (Fig. 2a).
24. Maintain the water of the system at a constant temperature similar to that at the collection place (between 20 and 25 °C).
25. Illuminate the animals with a low light on 12 h day and 12 h night photoperiod.
26. Pipette daily 2.5 mL of food mixture per 3 L of FSW.
27. Replace the FSW with fresh FSW every other day.
28. Wait 1 week after the collection for the colonies to attach to the glass slide.
29. Remove the cotton thread.
30. Transfer the new colonies to the main husbandry system.

3.2 DNA Barcoding

Total DNA isolation uses handmade buffers adapted from a previously published protocol [33].

1. Centrifuge the sample to be used for DNA barcoding for 2 min at max speed.
2. Discard the supernatant and leave it under a hood until ethanol completely evaporate (~1 h).
3. Add 150 µL of lysis buffer.
4. Homogenize with a single use pestle.
5. Add another 150 µL of lysis buffer.
6. Add 60 µL $NaClO_4$ stock solution.
7. Add 360 µL of phenol–chloroform–isoamyl alcohol.
8. Vortex the tube for 10 min at 600 rcf in a hood.
9. Centrifuge for 10 min at 14,000 rcf, 4 °C.
10. Transfer the upper phase (around 350 µL) in a new 1.5 mL tube.
11. Add 350 µL of chloroform–isoamyl alcohol to the isolated phase.

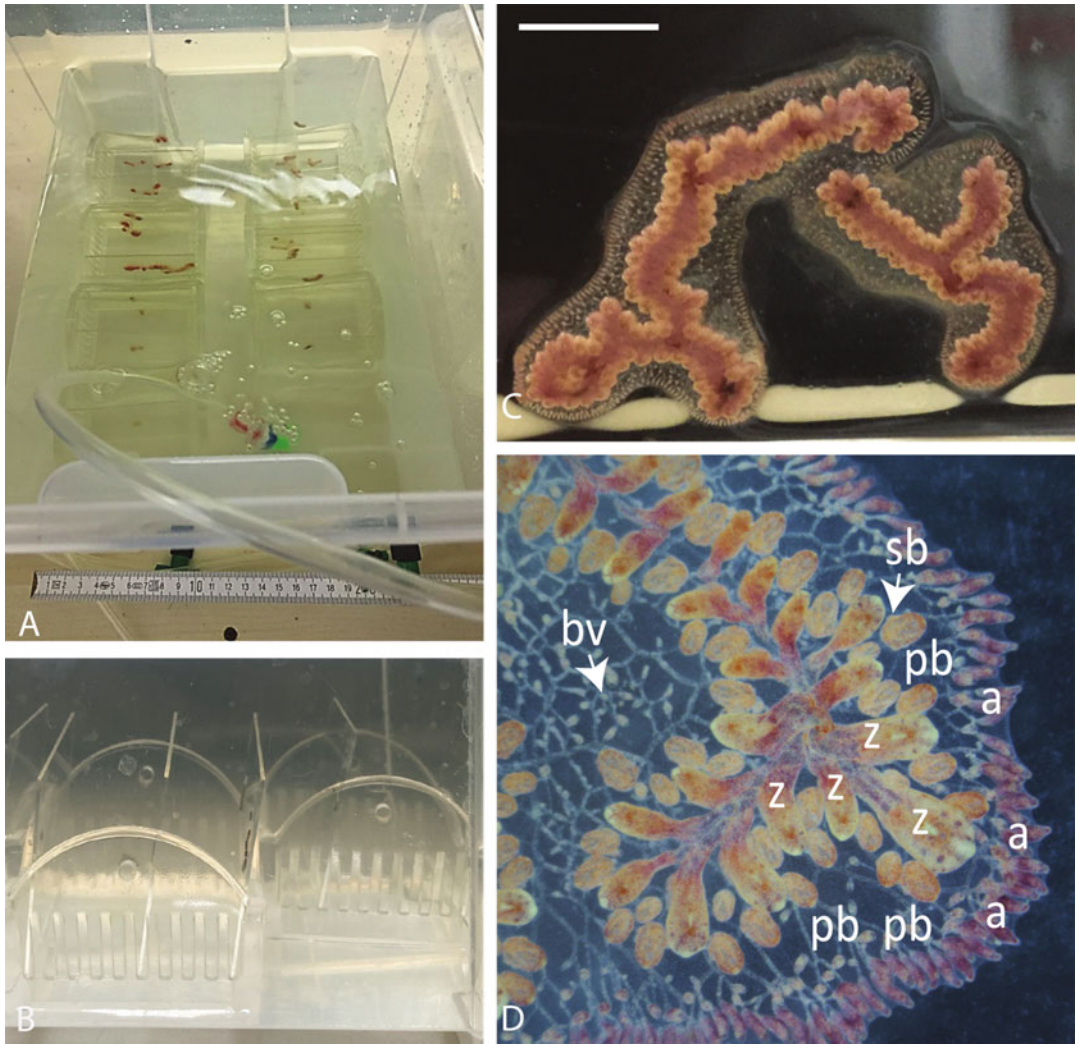

Fig. 2 Colony maintenance. (**a**) A *B. anceps* husbandry setup in our aquaculture room. (**b**) A side-view of a staining rack with *botryllid* colonies in it. (**c**) A top view of a *Botrylloides anceps* colony. Scale bar is 1 cm. (**d**) Magnification of a part of a system during the "takeover" (main zooids are being resorbed while the primary buds replace them). *a* ampulla, *bv* blood vessel, *pb* primary bud, *sb* secondary bud, *Z* zooid

12. Mix gently by shaking for 30 s.
13. Centrifuge for 10 min at 14,000 rcf, 4 °C.
14. Transfer the upper phase in a new 1.5 mL tube.
15. Add 350 µL of cold 100% EtOH to the isolated phase and mix gently (*see* **Note 13**).
16. Centrifuge the sample for 30 min at 16,000 rcf, 4 °C.
17. Replace the supernatant with 700 µL of cold 70% ethanol.
18. Centrifuge the sample for 30 min at 16,000 rcf, 4 °C.

19. Discard the supernatant.
20. Leave the sample under a hood overnight to dry.
21. Resuspend the DNA in 50 μL of ultrapure water.
22. Quantify the DNA concentration and quality using a spectrophotometer or equivalent tool (e.g., Qubit fluorometer).
23. Make sure the quality of DNA is within acceptable range (*see* **Note 14**).
24. Dilute the DNA with ultrapure water to reach a concentration between 10 to 100 ng/μL (*see* **Note 15**).
25. Transfer 25 μL of 2× PCR mater mix to a PCR tube.
26. Add 2 μL of DNA template.
27. Add 1 μL of cytochrome oxidase I primer mix.
28. Add 23 μL of ultrapure water.
29. Run a PCR program with the following parameters: 95 °C 5 min; 35 cycle × (95 °C 45 s; 45 °C 45 s; 72 °C 90 s), 72 °C for 10 min and infinite hold at 12 °C.
30. Load 4 μL of the PCR product with 1 μL of loading dye into a well of agarose gel.
31. Load 2 μL 100 bp DNA ladder into another well.
32. Run the electrophoresis for 60 min at 100 V.
33. Visualize the results of the PCR amplification on the gel (*see* **Note 16**).
34. Identify PCR positive samples by the presence of a 600 bp amplification band in the visualized gel by comparison with the DNA ladder.
35. Use a PCR clean-up kit to purify the positive PCR products (*see* **Note 17**).
36. Perform the Sanger sequencing for both directions (forward and reverse) of the purified COI amplifications (*see* **Note 18**).
37. Trim the primer sections from the raw sequencing results (*see* **Note 19**).
38. Run your trimmed sequences through the BOLD Identification System [34] (*see* **Notes 20** and **21**).
39. Record the Probability of Placement to determine the species of your sample (*see* **Note 22**).
40. Assign your sample to the species with a sequence match of 97% or above and a query cover of at least 85% (*see* **Note 23**).
41. Submit your DNA barcode to the BOLD with all the information about sampling areas, specimen and if possible, a voucher ID from a fragment of your sample deposited in a museum (*see* **Note 24**).

3.3 Colonies Maintenance

Our maintenance protocol has been adapted from the work by Rosner et al. (2019) [35].

1. Pipette daily 2.5 mL of food mixture per 3 L of FSW.
2. Replace the FSW with fresh FSW every other day.
3. Once a week, transfer the colonies to a smaller container filled with FSW for maintenance.
4. Prepare a humidity chamber for the subcloning by placing a glass staining rack onto a wet tissue inside a sealable container.
5. Take one slide out of the water.
6. Clean the glass slide around the colony by scratching it using a razor blade.
7. Gently brush the colony with a soft paint brush to remove debris and dirt.
8. Check the colony under a stereomicroscope (*see* **Note 25**).
9. Ablate portions of the colony that are degrading, dirty or unattached to the glass slide.
10. If the cleaned colony is smaller than 2 cm in diameter, put it back into the husbandry system.
11. For larger colonies, detach a small piece of the main colony containing between 5 and 10 zooids using a razor blade (Fig. 2c, *see* **Note 26**).
12. Transfer the fragment (called subclone) onto a new glass slide using a brush.
13. Gently dry out the water around the colony fragment without touching the animal using paper tissue.
14. Give a unique identification code to the fragment.
15. Write the code on the glass slide.
16. Place the slide in the humidity chamber.
17. Wait 30 min for the colony to attach onto the slide.
18. Place the new slide carefully into the husbandry system.
19. Repeat **steps 5** to **18** until all slides have been processed.
20. Handle the new slides very carefully during the first week.

3.4 Whole-Body Regeneration

Our regeneration protocol has been adapted from the work by Rinkevich et al. (2007) [36].

1. Transfer *B. anceps* sub-clones into a container filled with FSW (*see* **Note 27**).
2. Place a slide upside-down under a stereomicroscope.
3. Observe the buds and budlets (the buds' buds) to determine the blastogenic stages (Fig. 2d, *see* **Note 28**).

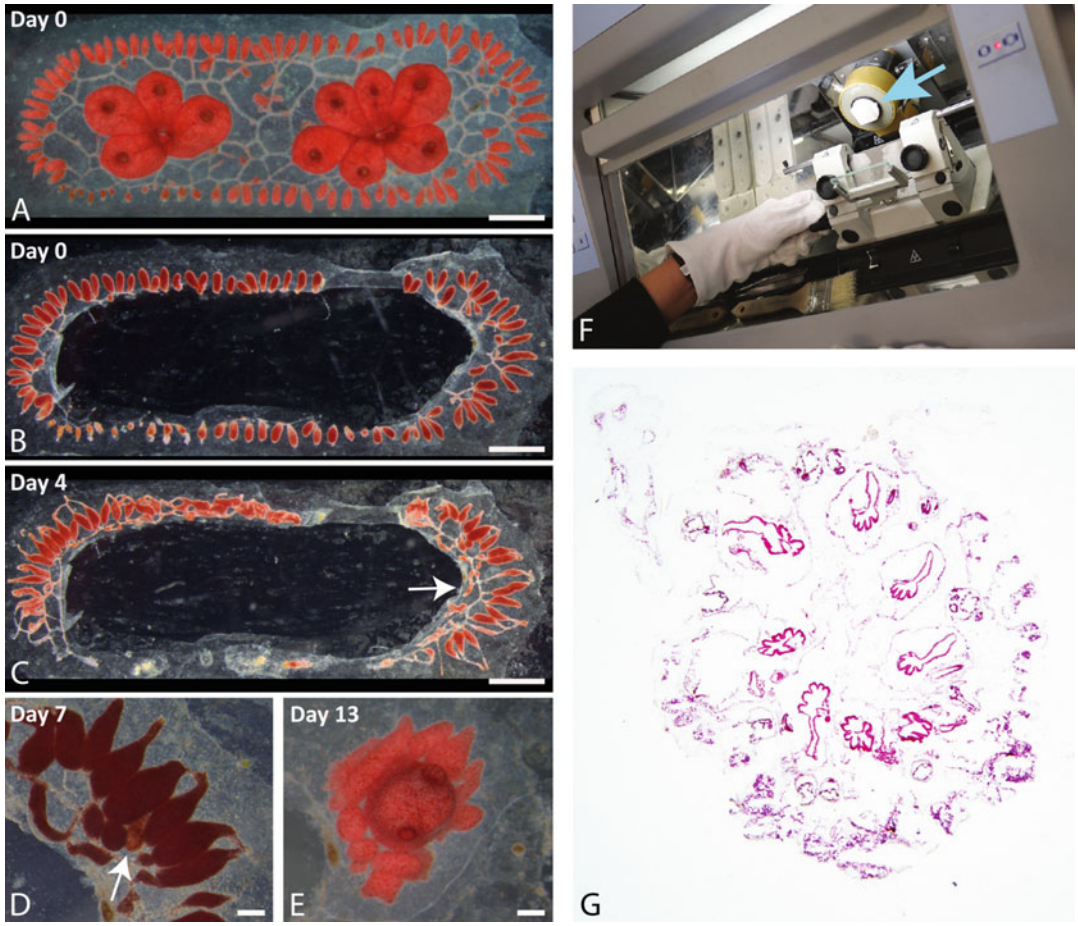

Fig. 3 Whole-body regeneration. (**a**) A *B. anceps* colony before removing zooids and buds. Scale bar is 1 mm. (**b**) The same colony after removal all the zooids and buds. (**c**) Fourth day post ablation. (**d**) Magnification of a portion of the regenerating colony where a regeneration niche is developing (arrow). Scale bar is 200 μm. (**e**) Fully completed WBR (day 13th). (**f**) The frozen mold (blue arrow) attached to the cryostat for sectioning. (**g**) A section of *B. anceps* colony at 1.2× magnification

4. Turn the slide to have the colony facing upwards.
5. Take a picture of the whole colony together with a scale (Fig. 3a).
6. Remove all the zooids and buds by carefully cutting all the blood vessels between the zooid, buds and ampullas with the pointy side of a fine needle, leaving only ampullas and peripheral blood vessels (Fig. 3b).
7. Record the date and time.
8. Transfer the regenerating colonies to a dedicated husbandry system.
9. Do not feed them (they will not take it) but change the water with fresh FSW every other day (*see* **Note 29**).

10. Wait until the desired stage of WBR is reached (*see* **Note 30**).
11. Take pictures from both sides of the regenerating colonies (Fig. 3a–e).
12. Proceed with the desired downstream analysis.

3.5 Histological Sectioning

Although the most common protocols for histology use paraffin embedding, we prefer to use cryosectioning for its quick turn-around time.

1. Transfer the colony to be fixed into a container filled with 100 mL of FSW.
2. Add a few menthol crystals to the water.
3. Wait 10 min for the colony to anesthetize.
4. Detach the colony from the glass using razor blade.
5. Transfer into a 2 mL tube filled with 1.5 mL of Bouin's fixative (*see* **Note 31**).
6. Fix overnight at 4 °C.
7. Replace the fixative with PBS.
8. Discard the rinsing medium.
9. Fill the tube with PBS.
10. Wait 2 h for the sample to wash.
11. Repeat **steps 8** to **10** three more times (*see* **Note 32**).
12. Transfer the sample to a 2 mL tube filled with 1.5 mL dehydration solution.
13. Leave 1 h at 4 °C to dehydrate (*see* **Note 33**).
14. Precool a metal block in a closed container filled half-way with liquid nitrogen (*see* **Note 34**).
15. Fill a labeled embedding mold with OCT (*see* **Note 35**).
16. Transfer the colony into the mold using a pair of tweezers (*see* **Note 36**).
17. Center the colony at the bottom of the mold with a pair of tweezers (*see* **Notes 37** and **38**).
18. Place the mold onto the cold metal block.
19. Wait 5 min until it becomes fully opaque.
20. Store up to 12 months at −80 °C until sectioning.
21. Transfer the block into the cryostat.
22. Peel the mold to release the block of frozen OCT.
23. Glue the block onto the cryostat's sample holder using a few drops of OCT.
24. Freeze the sample holder.
25. Label 10 coated microscopy glass slides (*see* **Note 39**).

26. Fix the sample holder onto the cryostat (Fig. 3f).
27. Cut the excess OCT around the sample using a razor blade (*see* **Note 40**).
28. Bring the sample close to the blade.
29. Section until the blade cuts the block twice.
30. Move the blade backwards.
31. Realign the sample in the direction of the last cut to place it perpendicular to the blade (*see* **Note 41**).
32. Repeat **steps 28** to **31** until the cryostat cuts through the whole surface of the block (*see* **Note 42**).
33. Cut a section of the block (*see* **Note 43**).
34. Transfer it to the next available position on the slides (*see* **Note 44**).
35. Brush the blade to remove debris and ice.
36. Brush the sample to remove leftover OCT.
37. Repeat **steps 33** to **36** until no sample is visible in the last section.
38. Wait 20 min for the slides to dry.
39. Clean the cryostat following the manufacturer's instructions.
40. Store the dried sections at −20 °C until needed.

3.6 Hematoxylin-Eosin Stain

1. Thaw the cryosectioned slide on the bench (*see* **Note 45**).
2. Transfer the slides into a Coplin jar filled with tap water (*see* **Note 46**).
3. Leave the section to rehydrate for 5 min.
4. Transfer to another Coplin jar filled with Groat's hematoxylin.
5. Stain for 12 min (*see* **Note 47**).
6. Shake vigorously each slide in the differentiation solution for 20 s.
7. Transfer into a Coplin jar filled with tap water.
8. Place the Coplin jar in the sink under a stream of running tap water.
9. Leave 10 min to color.
10. Rinse the slides in distilled water.
11. Transfer into a Coplin jar filled with Eosin.
12. Stain for 5 min.
13. Rinse the slides in 80% EtOH.
14. Transfer the slides to 100% EtOH (*see* **Note 48**).
15. Wash for 3 min.

16. Transfer to a bath filled with clearing agent.
17. Wash for 3 min.
18. Repeat **steps 16** and **17** twice.
19. Place a slide flat on a paper tissue.
20. Pour 3 drops of mounting medium over the sections.
21. Hold a sufficiently long coverslip over the stained sections.
22. Rest one side of the coverslip on the corresponding side of the slide, still holding the other side.
23. Hold the other side of the coverslip using a short needle placed under the coverslip.
24. Slowly lower the coverslip until it rests fully flat on the slide (*see* **Note 49**).
25. Identify bubbles trapped under the coverslip.
26. Swipe the air out of the mounting medium by pressing on the coverslip with the needle while holding the coverslip in place with the other hand (*see* **Note 50**).
27. Cover the mounted slide with a paper tissue.
28. Gently press uniformly on the coverslip to remove any excess mounting medium.
29. Leave the slide to dry for 30 min.
30. Use a paper tissue dipped in clearing agent to clean the dried mounting medium on the slide.
31. Image the slide under a brightfield microscope.
32. H&E sections will have dark DNA stains and purple to red cytoplasm (Fig. 3g).

4 Notes

1. These primers have been designed specifically for botryllids [37]. Universal invertebrate primers (Forward: GGTCAA CAAATCATAAAGATATTGG, Reverse: TAAACTTCAGGG TGACCAAAAAATCA, [38]) and tunicate specific primers (Forward: TCGACTAATCATAAAGATATTA, Reverse: AAC TTGTATTTAAATTACGATC, [39]) can also be used using the same protocol.
2. Handle the saturated solution of picric acid very carefully and do not let it dry in the bottle as picric acid crystals are explosive! To circumvent this issue, we favor the use of commercially available Bouin's fixative.
3. Although *B. anceps* colonies were the only species found in some locations, they more typically share their habitat with

other invertebrates (mostly sponge) and other botryllids (e.g., *Botryllus schlosseri, Botrylloides aff. leachii, Didemnum* sp.). All of them can be collected and kept in the aquaculture room using the same protocol.

4. Sometimes the species cannot be found even in very suitable rocky habitat. You may need to check many coastlines to find this particular species.
5. The salt concentration is one of the main requirements for thriving botryllid ascidian culture. Both artificial and natural seawater can be used. In our institute, we are using natural seawater collected on the Mediterranean Sea coastline. However, likely because of the presence of multiple freshwater streams in the area, we have observed that the salinity on our shores is not stable. To circumvent this issue, we measure the salinity of each new batch of FSW, correct it by adding sea salt and keep it in our aquaculture room for 1 week before use.
6. To preserve the biodiversity, we recommend to collect only part of each identified colonies, typically around 1 mm of zooids. Consequently, only colonies larger than 2–3 mm long are suitable for collection.
7. *B. anceps* colony is prone to folding during the sampling. Make sure that once on the glass slide, all zooids of the colony are facing upwards. Like on the stone, their atrial and buccal siphons should be facing towards the water and not towards the glass.
8. This technique is adapted from a previously published protocol for the collection of colonial ascidians [40].
9. We recommend placing only three slides on each rack, to give them enough place for an effective water circulation.
10. Species identification is one of the main issues when working with botryllid ascidians. Indeed, many species have a similar external morphology, color patterning is not a good discriminant and chimerism is common in Botryllid ascidians. Consequently, DNA barcoding should be used to overcome this identification problem. Tissue fragment for DNA barcoding can either be isolated directly during sampling (Fig. 1d) or from a sub-cloned colony in the husbandry system.
11. There is no need to use air stone while transferring the animals to the lab.; they can survive hours without oxygen support.
12. Botryllid cultures are typically done in plastic container. These vessels are easy to handle, to carry, and to wash. However, prefer food-grade containers to avoid chemicals leaking in the water.

13. You should see the DNA precipitating as white strings. If you do not see any precipitation, store the sample overnight at −20 °C to increase the precipitation.
14. In the nanodrop spectrophotometer, the value 1.8 from 260/280 ratio represents the pure DNA and 2.0–2.2 from 260/230 ratio pure nucleic acid. Be sure your DNA in this range or close these values. If not, you may need to isolate the DNA from the beginning (if you have some remaining sample) or clean the isolated DNA by bringing the volume to 100 μL by adding lysis buffer and restart from **step 4**.
15. Dilution of the DNA is necessary because high DNA concentrations inhibit the reaction. Besides, it may yield big shining plums (smears) of nonamplified DNA scattered along the agarose gel.
16. If you record double PCR products for one sample, set the PCR again, and rearrange the annealing temperature between the 46 to 49 °C, it will help reduce nonspecific PCR products.
17. After a PCR reaction, purification of the targeted genes is necessary for downstream use, and it facilitates removing enzymes, nucleotides, detergents, primers, and buffer components.
18. Performing the sequencing to both directions allows you to double-check your sequences by reducing nucleotide assignment errors.
19. Without trimming, you may get less coverage from the blast analysis. So, we highly recommend trim off the primer sites and use only the central sequence.
20. The NCBI BLAST engine can be used as alternative or as complement to the BOLD identification system [41].
21. It is also possible to use these sequences to build a reference DNA barcode library for a known species as identified by an expert taxonomist. This approach has the advantage of providing a trustful reference to the entire community.
22. Probability of Placement (%): how similar the query sequence is to the target sequence, that is, how many characters in each sequence are identical. The higher the percent identity is, the more significant the match.
23. Be aware that there are some mislabeled sequences in public reference databases [42]. In the case your sequence matches several different species, favor the references uploaded by expert taxonomists.
24. If you encounter a big enough colony during the sampling, take the third fragment beside the aquaculture and DNA barcoding's processes and preserve it into a 10% formalin solution

Table 1
Staging method of the blastogenic cycle

Stages	Features
A	It starts with the opening of the buccal and atrial siphons
B	Initiates with the beginning of heartbeats in the primary buds
C	It begins with the onset of organogenesis in secondary buds and the accumulation of pigment cells in the primary buds' epithelium
D	Commences with the shutdown of buccal and atrial siphons

and submit it in a museum with a unique voucher ID. Load this ID to NCBI or the BOLD system when you upload your barcodes.

25. Sometimes large number of Ciliates can be observed on the slides. In case of such invasion, transfer the concerned colonies to a temporary container, wash all the husbandry system, glass racks and brushes with 10% (v/v) bleach. Clean the dead parts of each colony with a single edge razor blade and a soft brush using fresh seawater. Finally, transfer the cleaned colonies into a new husbandry system.
26. When a colony covers more than 1/4 of the slide, make sub-clones with half of its zooids and put the resulting new slides in a new husbandry system.
27. We recommend starting a WBR experiment with at least 8 colonies from the same original clone to provide sufficient material for histological sectioning and biological replica.
28. We divide the blastogenic cycle into four major stages as previously published [43]. The details about each stage are given in Table 1.
29. During WBR there are no zooids left to consume the food. The decaying food will contaminate the entire container.
30. Monitoring WBR progression at least on a daily basis. Record the time of observation precisely to match it with the defined stages of WBR ([44], Table 2). Alternatively, film the whole processes with a high-quality camera to monitor WBR.
31. Fixing the colonies at different stages of WBR for immunohistochemistry analysis will help to observe this process at the cellular and sub-cellular level [15].
32. Large samples might require more washing steps. Repeat washing until the PBS has a no more yellow taint.
33. Dehydration time depends on the size of the sample. Dehydration is complete when the sample sinks in the sucrose.
34. A block of dry ice can also be used instead of the cooled metal block.

Table 2
The whole-body regeneration stages of *B. anceps*

Stages	Time	Features
Stage 0	0–30 m	Hemorrhagic period; after removing all the zooids and buds, hemorrhagic period stopped in 1 min (Fig. 3b)
Stage 1	30 m–24 h	Formation of the new vessels (Fig. 3b)
Stage 2	48 h–120 h	Reorganization; the tissue underwent considerable restructuring as it condenses together inside the tunic (Fig. 3c)
Stage 3	144 h–168 h	Formation of the regeneration niches (Fig. 3d)
Stage 4	192 h–384 h	Formation of the functional zooid (Fig. 3e)

(*m* minute, *h* hours)

35. It is very important to avoid bubbles at this step. The best approach to do so is to pour the OCT in one continuous streak in one corner of the mold. In case bubbles get trapped in the OCT, group them at the surface of the OCT in one corner of the mold.
36. Do not squeeze the sample during this transfer or it will get damaged.
37. Alignment of the colony is typically done with its tunic lying flat at the bottom of the mold and its zooids facing upwards. Other alignments to suit different types of histological sections can easily be produced using the same approach.
38. OCT is highly viscous so moving the submerged sample can be a bit difficult as any movement of the tweezers will move it. We advise to push the sample using a pair of tweezers and to use how much they are open to modulate how gentle the strokes are.
39. The exact number of slides that your sample will occupy depends on the thickness of your sectioning and of your sample. In our hands, for relatively flat colonies and a sectioning at 12 μm, 10 slides were optimal. We also tested 12 slides at 10 μm and 8 slides at 14 μm.
40. Removing OCT around the sample allows to have more sections on the same glass slide, which is very useful for downstream analyses. However, removing too much OCT will cause the sections to be teared during sectioning. We recommend having at least 1 mm of OCT surrounding the sample.
41. The direction and the amplitude of the realignment will need to be adapted to your cryostat and your current sample. Make

sure that the blade is secured and the sample is blocked before you start moving it.

42. Be aware that for blocks with colonies aligned at the bottom of the block, this realignment should be done efficiently as you will start cutting into the sample almost immediately.
43. Obtaining good sections requires a bit of practice. The sharpness of the blade is obviously very important, but the speed and fluidity of the movement of the sample are crucial too. Typically, we advise moving the sample in one continuous movement that lasts about 2 s. Some of the main issues observed during sectioning are that sections can be teared, they can fold, they can curl or they can stay attached to the main block. In the first case, try varying the speed of the cut. If that does not suffice, sample might have required a stronger fixation or embedding included small bubbles. Increasing the thickness of the sections is the most effective solution to this problem. For the folding, try to clean the blade some more as even very small debris can prevent the section from sliding properly on the blade. Curling was mainly observed when room temperature and humidity was too high. Curled sections can be flattened using a thin paint brush. However, curling takes a few seconds to occur so we rather recommend that the transfer be done very quickly. Attachments are typically due to excess OCT at the top of the block. Brushing out this material should solve the issue. If not, a small bezel cut with a razor blade to remove this upper edge should be very effective.
44. We use two main organizations for the sections. First a continuous one, where each section follows the previous one. This has the advantage of being very flexible in terms of the number of slides that are used. Second, a parallel one, where all the slides are filled in parallel. This has the advantage of yielding slides that are almost perfect replica of one another. This approach is particularly useful for comparing different stainings.
45. Thawing typically takes around 2 min. Wait until all condensation has evaporated from the slides.
46. The minerals present in tap water are beneficiary to the staining process. For the specified steps, better results were obtained with tap water than with deionized/distilled water.
47. Although H&E is one of the most straightforward and reproducible histological stain, the intensity of the stain could be influenced by many parameters including the thickness of your section, the type of OCT or the fixation of your sample. It might thus be necessary to modify the duration of the staining steps.

48. At this step, the slides could be checked under a microscope for the quality of their staining. Staining of both hematoxylin and eosin could be increased by repeating the corresponding steps.
49. Mounting medium are typically highly viscous. It is thus necessary to lower the coverslip slowly to avoid trapping air bubbles under it.
50. The point is to push the air bubbles toward the boundaries of the coverslip by pressing around it and following it as it moves. Note that only bubbles in contact with the sample will be an issue during imaging. Other bubbles could be left where they are.

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