Chapter 12

Collecting and Culturing *Lineus sanguineus* to Study Nemertea WBR

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Abstract

Whole-body regeneration, the ability to reconstruct complete individuals from small fragments, is rare among ribbon worms (phylum Nemertea) but present in the pilidiophoran species *Lineus sanguineus*. This species can regenerate complete individuals from a tiny midbody section, and even from a quarter of a piece, provided it retains a fragment of a lateral nerve cord. While a few other unrelated species of ribbon worms are also excellent regenerators, *L. sanguineus* is unique in having evolved its regenerative abilities quite recently and thus offers an exceptional opportunity to gain insight into the evolutionary mechanisms of regeneration enhancement. Interestingly, both its sister species *Lineus lacteus* and *Lineus pseudolacteus,* a third species derived from the recent hybridization of the other two, differ in their regeneration abilities: while *L. lacteus* is uncapable of regenerating a lost head, *L. pseudolacteus* is capable of anterior regeneration, albeit at a slower rate than *L. sanguineus. L. sanguineus* has a worldwide distribution in temperate shores of both hemispheres, is readily found at intertidal habitats, and can survive, feed and be bred through asexual replication with minimal effort in laboratory settings. All the above make this species a superb candidate for studies of regenerative biology. In this chapter, we present protocols to collect, identify and breed *L. sanguineus* to study the extraordinary whole-body regeneration abilities found in this species.

Key words Heteronemertea, Intertidal, Invertebrate rearing, Pilidiophora, Spiralia

1 Introduction

Regeneration, the ability of organism to regrow lost body parts, is widespread across metazoan groups [1–3]. Regeneration varies broadly both in restorative potential and phylogenetic distribution: while many lineages are only capable of physiological tissue turnover or restoration of smaller amounts of lost tissues, others are capable of amazing regeneration feats, from restoring lost appendages to reconstructing whole new individuals from very small fragments. This later ability, known as whole-body regeneration, is well exemplified by planaria and other turbellarian flatworms, but is also commonplace in many cnidarians, ctenophores, sponges, xenacoelomorphs, and colonial tunicates. Some other groups, such as annelids and echinoderms, also show members with more

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limited but still exceptional regenerative powers [3]. Such broad array of regenerative potential across animals suggests that regenerative abilities have a rich evolutionary history that is mostly unexplored.

Understanding how regeneration evolves can help elucidate the cellular and molecular underpinnings of this developmental ability. One particularly informative approach is through comparative studies of regenerative ability in species that span an evolutionary transition in regenerative potential. Candidate mechanisms can be gleaned from comparing developmental and molecular genetic differences across such species and correlating them with changes in extent of regeneration. Mechanisms can then be experimentally assessed to test whether they inhibit or enhance regeneration. Studies on species spanning an evolutionary loss or reduction of regenerative ability can inform how the potential to regenerate might have become blocked or dampened, and eventually lead to strategies to lift or alleviate such blocks and allow for better regeneration and healing in systems that do not regenerate well (including most mammals in general, and humans in particular). In contrast, studies on species spanning an evolutionary gain or enhancement of regenerative ability can give insight on how organisms might be able to reboot embryonic developmental capabilities in a postembryonic context and inspire novel tools to induce regeneration after traumatic injury. While many evolutionary transitions leading to diminished or lost regenerative ability have been identified, there are very few examples of increased or gained regeneration [4, 5]. Thus, while we have many systems where we can study how regeneration is lost, we lack good models of how it is gained.

Ribbon worms (phylum Nemertea) are a phylum of about 1300 known species of elongated, primarily marine predatory worms [6–8]. While most species of nemerteans are capable of restoring a lost posterior end after a transverse amputation behind the brain, only a few have so far been shown to be able of restoring their anterior end after a similar injury [9]. The few species capable of anterior regeneration are taxonomically scattered across the phylum; ancestral trait reconstruction strongly suggests that lack of anterior regeneration is the ancestral and most common condition for nemerteans. Thus, species capable of regrowing a lost anterior end represent lineages that experienced evolutionary gains in regenerative ability.

Among anteriorly regenerating species, *Lineus sanguineus* (Rathke, 1799) stands out, unquestionably one of the champions of regeneration possessing some of the highest regenerative abilities known among animals [2]. A single worm of this species can be repeatedly amputated to obtain a complete regenerated worm just 1/200,000th of the volume of the original individual. Furthermore, a complete worm can regenerate not only from a thin

transverse slice of the body, but even from just one *quadrant* of a thin slice [10]. Regeneration rate varies with the size and condition of the fragment, but a recognizable head and tail can be rebuilt in around a week or two.

Lineus sanguineus individuals tend to have a slender body, often a 100 times longer than their body width (Fig. 1a). They are slightly flattened dorsoventrally with a pair of long lateral grooves at the anterior end, followed by a reddish brain region. They have about 2 to 8 pairs of dorsolateral ocelli arranged in a bilateral pair of rows extending along the anterior half of the head, over the lateral cephalic slits. The mouth opens ventrally some distance behind the brain. Except for the frontal margin and lateral borders of the head, worms tend to be uniform in color. Color itself varies across the species' distribution, showing olive, green, brown or red hues. *L. sanguineus* inhabits sheltered stony regions, among algae, lurking within shellfish beds, or inside the fouling/encrusting community growing over natural and manmade substrates of the intertidal zones of the marine shore. It can be found on temperate seashores around the world [9, 11–15] (Fig. 1d). At the Southern Hemisphere, it has been found on the South Atlantic shores of Uruguay and Argentina, and the South Pacific shores of Chile and New Zealand. In the Northern Hemisphere, it has been reported from the North Atlantic shores of North America (from the Gulf of Mexico and Florida to Newfoundland, Canada), Europe (along the Bay of Biscay, English Channel and North Sea) and Eastern Asia (South and East China Seas, Yellow and Bohai Seas, Sea of Japan and eastern Japanese Atlantic shores). This widespread distribution makes it more readily available for collection by researchers near most temperate locations around the world.

Lineus sanguineus belongs to the class Pilidiophora, the nemertean group that contains the highest number of species with whole-body regenerative ability [9]. This cosmopolitan species [12] belongs to a mostly European species group that also includes *Riseriellus occultus* (described from NW Spanish and N Welsh shores), *Lineus longissimus* (found all along European Atlantic shores), two cryptic species known as *Lineus lacteus* (*L. lacteus A*, associated with the Bay of Biscay and English Channel shores, and *L. lacteus M*, found in the Mediterranean Sea), and the endemic *Lineus pseudolacteus* (found only at the French Atlantic shores near Roscoff, Bretagne) [12, 13]. Of the above species, only *L. sanguineus* and *L. pseudolacteus* are capable of anterior regeneration. The other species are limited to regenerate posterior ends [9].

Lineus sanguineus and *L. lacteus A* are sister species, estimated to have diverged about 10 My ago, while *L. pseudolacteus* likely emerged from a much more recent (12–25 Ky ago) single hybridization event between *L. sanguineus* and *L. lacteus*—likely after fertilization of an unreduced *L. sanguineus* oocyte by a *L. lacteus* sperm [13, 16]. Due to its triploid condition, *L. pseudolacteus* has

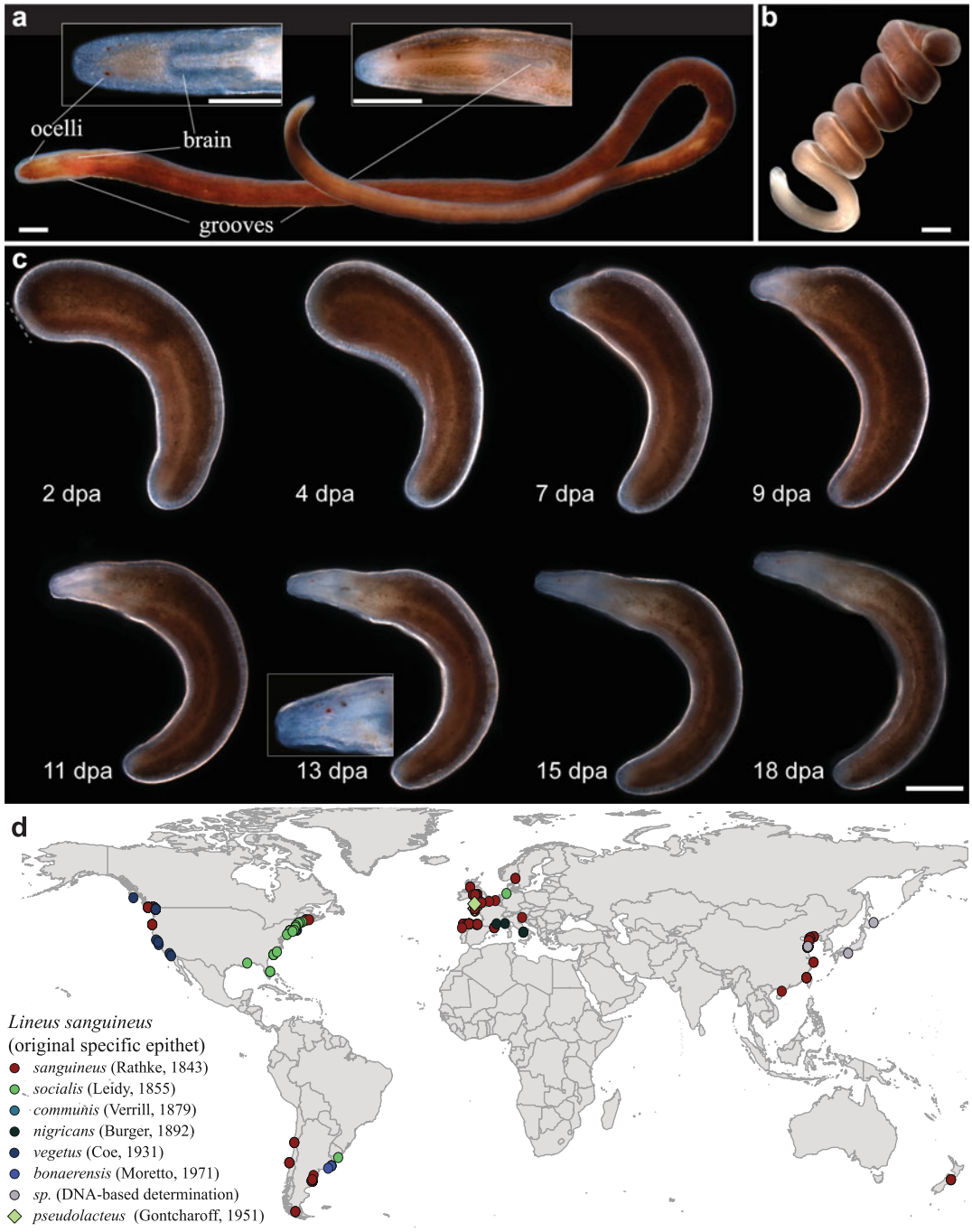

Fig. 1 Live and regenerating examples of *Lineus sanguineus*. (**a**) Live, extended individual of *L. sanguineus*; this specimen is relatively short—much longer specimens can be found. Anterior end at the left. The left inset shows a detail of the head in dorsal view. The right inset shows a detail of the head on lateral view (**b**) Live, coiled individual of *L. sanguineus*. Head toward upper right (**c**) Example of anterior regeneration from a posterior fragment, shown from 2 through 18 days postamputation (dpa); the red dashed line at 2 dpa shows the location of the healed anterior wound. Notice first ocelli appearing at 7 dpa, proboscis apparatus formed by 11 dpa and brain visible by 13 dpa (inset showing detail of head on lateral view); also note how the stump elongates and becomes slenderer to match the width of the regenerating anterior end. (**d**) Current known

been reproducing exclusively asexually since that event, a regeneration-dependent strategy inherited from their maternal species. Lack of anterior regeneration in *L. lacteus* places an upper bound on how long ago did the *L. sanguineus* lineage evolve anterior regeneration. Both parent species have been shown to possess private alleles, that is, unique haplotypic variants found only in one of the species. This should give a considerable fraction of their genome a specific signature which could facilitate detection of allelic bias in genes differentially expressed during regeneration of *L. pseudolacteus*, complementing gene expression studies comparing postamputation responses between *L. lacteus* and *L. sanguineus*. Furthermore, populations of *L. sanguineus* display different morphotypes with corresponding differences in regeneration potential, that are not obviously correlated with genetic differences [9, 12, 13]. For example, two ecologically isolated morphotypes with the same genotypic structure are present in Iberian shores: smaller individuals (<5 cm of total length) can be found among algae in lower, mid and sub-tidal regions, while the larger ones (5–20 cm) are found among sand below boulders in the high intertidal region. Interestingly, larger individuals regenerate more slowly than the smaller ones, suggesting that regenerative potential might be modulated by physiological trade-offs and local adaptations. All the above make *L. sanguineus, L. lacteus* and *L. pseudolacteus* a unique and powerful three-species system to inquire into the molecular and developmental mechanisms that evolved to enable the spectacular whole-body regeneration currently found in *L. sanguineus*.

In this chapter, we present simple and inexpensive methods to collect, keep and experiment on *Lineus sanguineus*. Most of these methods are applicable also to *L. lacteus* and *L. pseudolacteus* (except of course for asexual propagation, which cannot be used in *L. lacteus*). Many of these methods might also apply to a variable degree to other nemertean species too.

2 Materials

2.1 Field Collection of Specimens

1. Several medium sized, light colored (preferably white) buckets or deep trays.
2. Gallon-sized bottle or can (one to several).
3. Quarter-gallon, sturdy zipper bags or similar.

Fig. 1 (continued) distribution of *Lineus sanguineus*. Occurrence data obtained from the Global Biodiversity Information Facility and other sources [9, 11–14, 25] and curated by the authors. Occurrences are color-coded to show the different species under which the specimens had been originally described; all but *Lineus pseudolacteus* are now synonymized to *Lineus sanguineus*

4. Small sturdy knife (preferably of rust-resistant steel).
5. Soft round art paintbrush.
6. Pasteur plastic pipettes.
7. Clean plastic or glass vials.
8. Hammer and chisel and/or spatula.
9. Magnifying glass (optional, used to examine smaller specimens more closely).
10. Insulated cooler filled with ice.

2.2 Specimen Rearing

1. Filtered sea water (FSW): 0.45 μm filtered either natural or artificial seawater (*see* **Note 1**).
2. Glass dessert bowls, crystallizer dishes or fingerbowls.
3. Petri dishes (glass or plastic) with a diameter larger than that of the bowls.
4. Labeling tape.
5. Calf liver: ~250 g, ideally from organic-certified sources.
6. 200–400 μm mesh for tissue homogenization.
7. Disposable syringe (without the needle).
8. Polyethylene film or Parafilm.
9. #10 scalpel blades and scalpel handle.

3 Methods

3.1 Field Collection of Specimens

Lineus sanguineus inhabit rocky or pebbly areas of the intertidal zone, the region left exposed by receding waters during low tides (*see* **Note 2**, Fig. 2a). They often co-occur with other, sometimes similar looking species (*see* **Note 3**), like *Lineus ruber*, *L. viridis*, or *L. clandestinus*. In some places and locations, worms can be found and collected directly behind rocks and pebbles. This method is typically suitable for large specimens from the high intertidal zone (*see* **Note 4**). In most other occasions, however, they lurk within encrusting and fouling communities growing over large rocks and cannot be easily retrieved directly. In those cases, the most successful strategy is to cause hypoxia-induced migration (*see* Subheading 3.2).

1. Wait for waters to recede from the collecting area (Fig. 2a).
2. Pick up boulders and smaller pebbles (*see* **Note 5**; Fig. 2b).
3. Carefully examine the lower surface and any fissures of the boulder to see if there are worms.
4. Inspect the sand or mud that was below the boulder.

Fig. 2 Field collection of nemerteans. (**a**) A typical intertidal area where *L. sanguineus* can be found. (**b**) Direct collection of specimens under pebbles, rocks and shells. (**c**) Large specimen of *Lineus lacteus* inside a mollusk shell while eating its owner; the anterior end is deep within the shell. (**d**) Removal of a sample of the fouling community encrusted on the pylons of a pier. (**e**) Removed rubble is placed in trays and covered with sea water. (**f**) Rubble is spread out on the bottom of the tray, and allowed to become hypoxic, forcing nemerteans to come out of their shelter and allowing their collection

5. If you find one or more worms (Fig. 2c), gently collect the specimens using a paintbrush (*see* **Note 6**). A knife can be used to extract specimens from fissures in the boulder.
6. Place the individual(s) in a vial with seawater (*see* **Note 7**).
7. Remove most ice from an ice-filled insulated cooler.
8. Place vials with worms in the cold cooler, keeping it between 4 and 15 °C.
9. Bring cooler with worms to the lab or rearing facility.

3.2 Hypoxia-Induced Migration

This method, proposed by Kirsteuer [17], is suitable for smaller specimens from the low and mid intertidal region inhabiting within the encrusting community formed by algae, mussels, barnacles or other creatures adhering to a hard substrate (natural rocks and outcrops, or manmade structures like pilons and jetties). This strategy induces them to migrate out to the open by falling oxygen concentrations.

1. Use a chisel, spatula or knife to remove part of the encrusting community from the substrate (Fig. 2d).
2. Place removed rubble into a bucket or sturdy zipper bag.
3. Fill the container with seawater.
4. Repeat 1 and 2 until filling several bags.

5. Fill an additional bucket with seawater from the collection location.
6. (optional) Fill quarter-sized cans or bottles with seawater.
7. Bring back rubble bags, cans/bottles, and/or buckets to the lab or other work area.
8. Set up several deep trays or buckets on a table or the floor.
9. Empty rubble bags on trays/buckets, spreading the rubble evenly into a layer not thicker than 15–20 cm from the bottom of the container.
10. Add seawater to trays/buckets until the water level is twice higher than the rubble layer (Fig. 2e).
11. Fragment rubble into smaller pieces with hands or the help of a chisel or a spatula.
12. Spread fragments on bottom (Fig. 2e, f).
13. Keep at room temperature.
14. Monitor trays for the following 24 h for worms coming out of the rubble and crawling up the walls of the recipient (*see* **Note 8**).
15. Pick the worms gently using plastic pipettes or a soft paintbrush and place them in vials.
16. Keep the vials with worms between 4 and 15 °C until rearing bowls are set up (see Subheading 3.3).
17. Check periodically the trays/buckets for additional worms emerging from the rubble, until no more worms emerge, or the water becomes foul and turbid (*see* **Note 9**).
18. Discard the water and rubble (*see* **Note 10**).

3.3 Specimen Rearing

Collected specimens can be kept in the laboratory for many months and up to several years with minimal maintenance (*see* **Note 11**).

1. Set up clean, dry culture bowls (*see* **Note 12**).
2. Label bowls using labeling tape with date and source of specimens.
3. Add tempered FWS to fill about one half of the bowl.
4. Move 5–20 specimens (*see* **Note 13**) from collection vials to the bowl using either a paintbrush or Pasteur plastic pipettes (*see* **Note 14**).
5. Examine the behavior of the transferred worms (*see* **Note 15**).
6. Cover bowls with either plate of a petri dish to reduce evaporation; lids should sit loosely and not form an airtight seal.
7. Store the bowls at a cool (12 to 18 °C) location protected from direct sunlight. A temperature-controlled, constant-humidity chamber or incubator is optimal.

8. Check bowls daily, and use a soft paintbrush or plastic pipette to move any worms that have crawled up the walls over the water level back underwater (*see* **Note 16**).
9. Replace culture water with fresh FSW at least weekly (*see* **Note 17**).

3.4 Feeding

Although many nemerteans, including *Lineus sanguineus* can survive for many months without feeding, keeping well-fed worms will improve overall condition, yield more reliable experimental results and allow increasing population numbers through clonal propagation (see Subheading 3.5). *L. sanguineus* is a voracious predator, and likely a scavenger too, and will feed readily once it learns the nature of its food. It can be fed a variety of items, including live annelids, processed liver, minced scallops or mussels as well as eggs/oocytes from other invertebrates. In this protocol, we detail preparation of and feeding with liver homogenate, since this is a well-established method used to rear and perform dsRNA- or drug-mediated interference experiments on the planarian *Schmidtea mediterranea* [18, 19].

1. Cut a 100–250 g slice of calf liver into small (~3 cm) cubes.
2. Trim all fat, blood vessels, and other connective tissues.
3. Grind cubes using mortar and pestle, a potato masher, or a blender. If using a blender, use a slow speed to avoid formation of bubbles.
4. Pass the ground liver through a sieve with a 200–500 μm mesh to remove coarse fragments, bubbles and other tissue remains.
5. Cut out pieces of polyethylene film so they fit flat in a freezer container.
6. Remove the plunger from a disposable syringe.
7. Load the liver homogenate into the barrel.
8. Replace the plunger.
9. Place a first sheet of polyethylene film on the bench.
10. Push the plunger to extrude a round, long strip of homogenate over the plastic film (*see* **Note 18**).
11. Repeat to lay parallel strips of liver until completely depleting the barrel of homogenate.
12. Reload the barrel and repeat **steps 8** to **11** until all area of the film is covered with parallel stripes of liver.
13. Put another piece of polyethylene film on top of the liver stripes.
14. Repeat **steps 7** to **13** until all liver homogenate is laid down.
15. Place the stack of strips in a freezer container.
16. Store the freezer container at −20 °C.

17. At feeding time, thaw approximately 1 cm of liver homogenate strips per worm to be fed.
18. Label as many glass dishes as there are culture dishes to be fed.
19. Fill the feeding dishes to about half with FWS.
20. Move worms from their culture dish to the corresponding feeding dish using a paintbrush or plastic pipette.
21. Divide the liver strip into small pieces no larger than the worms are thick.
22. Add 2–3 pieces per worm to each feeding dish (*see* **Note 19**).
23. Sink any floating pieces of liver to the bottom of the feeding dish with a plastic pipette.
24. Replace water in culture dishes with fresh FSW.
25. Keep worms to feed for 1–2 h in feeding dish (*see* **Note 20**).
26. Return worms to their respective culture dishes (*see* **Note 21**).
27. Clean the feeding dishes.
28. Check culture dishes every 12 h for the presence of feces, remove them with a pipette.
29. Change the FWS from the culture dishes about a day or two after feeding the worms.

3.5 Propagation

Lineus sanguineus are known to reproduce asexually in the wild, and will readily do so in culture conditions [20]. Since they are capable of whole-body regeneration, population numbers can also be expanded by cutting worms into several fragments. Each fragment will regenerate the missing ends and result in a complete, albeit smaller worm. This technique allows not only to quickly expand the number of individuals, but also to generate smaller individuals that are better fit for experimentation, whole mount immunohistochemical procedures and imaging.

1. Label one glass dish per worm to be cut (*see* **Note 22**).
2. Fill the dishes to about half of their volume with FSW.
3. Add cold (4 °C) FSW to a shallow petri dish plate up to about 5 mm.
4. Move the worm to be amputated into the cold petri dish (*see* **Note 23**).
5. Wait until the worm starts crawling and extends.
6. Using a #10 scalpel blade (or similar curved edge blade), make a single transverse cut at about one third of the total body length from the anterior end.
7. Move the anterior fragment into a new, labeled culture dish (see Subheading 3.3).

8. Use the scalpel to make a cut approximately 1 cm posterior to the anterior cut surface (*see* **Note 24**).
9. Move the ~1 cm worm fragment to a glass dish.
10. Repeat **steps 8** and **9** until the length of the remaining posterior portion of the worm is about one third of its original length.
11. Move the posterior portion of the worm to the same culture dish used in **step 7**.
12. Discard water from the petri dish.
13. Wipe clean the bottom of the petri dish with a paper towel.
14. Repeat **steps 3** to **13** for each worm to be amputated.
15. Transfer with a plastic pipette all healthy amputated fragments (*see* **Note 25**) to small culture bowls, using one bowl per glass dish.
16. Keep worms at room temperature (*see* **Note 26**).
17. Check culture bowls 4 h after cutting and remove any dead or dying fragments.
18. Examine fragments in culture bowls 24 h after cutting and remove any dead or dying fragments (*see* **Notes 25** and **27**).
19. Replace water from culture bowls with fresh FSW (*see* **Note 28**).
20. Repeats **steps 18** and **19** at 48 h, 72 h, and 96 h after amputation, and then every 2 days thereafter.
21. When fragments have regenerated into worms in which ocelli, brains, mouth and a proboscis can be seen (Fig. 1c; *see* **Note 29**), move the culture bowls to the rearing chamber (see Subheading 3.3).

4 Notes

1. Clean sea water is the main requirement for successful maintenance and rearing of *Lineus sanguineus* and other nemerteans. While most marine research stations are fitted with sea tables and a constant supply of natural sea water, other locations are unlikely to have such facilities. If located near the ocean, sea water can be procured from the shore, brought in tanks or bottles, filter-sterilized and stored. Worm cultures use relatively small amounts of water, so unless there are many specimens being kept, only occasional trips would be needed. If located inland, then procuring natural sea water might not be practical. In such a case, it is possible to use instead one of the many formulations for artificial sea waters sold for aquariums. However, formulations not always yield an artificial sea water

equivalent to the one which the worms are habituated and might even prove lethal to them. Specific formulations should be tested on one or two individuals, by passing the worms through a graduated replacement from the natural seawater in which they were placed after collection to the artificial sea water. Even if the worms appear to survive the artificial medium, specifics of the formulation could affect regenerative ability. Thus, if setting up worm cultures for a research project at an inland location, it might be advisable to bring natural seawater from the collection sites and test that regenerative responses are similar in both natural and artificial media.

2. Several other species of nemerteans also inhabit the same habitats in which *Lineus sanguineus* can be found. Which species may be found will depend on the geographic location. Most of them can be collected and kept in the laboratory using the same methodologies described here.
3. Since several co-occurring *Lineus* species vary in coloration depending on the geographic region, sometimes determining if *L. sanguineus* is present may not be obvious. A useful behavioral test is to gently poke or disturb the worm with a jet of water from a pipette: while most species will contract their longitudinal muscles, becoming shorter and broader, *L. sanguineus* instead contracts diagonal muscles and coils into a spiral (Fig. 1b). If further confirmation is desired and resources are available, it is advisable to collect all specimens, bring them back to the lab and use molecular barcoding tools. Since most nemertean species readily regenerate lost posterior ends, the posterior end of each worm can be amputated and subject to DNA extraction without sacrificing the whole specimen. The barcoding fragment of cytochrome oxidase unit I (COI) can then be amplified using Folmer's L/H primer pair [21], sequenced and compared against other nemertean sequences deposited at NCBI's GenBank database (https://blast.ncbi.nlm.nih.gov).
4. Best collection sites are those with ample intertidal zones. Plan collection trips ahead of time, and always consult the tide tables for the area you will be collecting. Begin fieldwork well ahead of the time the tide will reach its low point, as waters are receding. Do not attempt to collect at a spot that is still close to where waves are breaking, and always retire from the area once the tide begins to rise. If you are new to the area, it is always advisable to try finding advice or help from someone with experience on local tide patterns.
5. Worms can also be found hiding or feeding inside mollusk shells, or even inside live or freshly dead mollusks and other animals.

6. Nemerteans are prone to breaking into pieces either during collection, or when placed in stressful conditions. Usually, the fragment containing the anterior end of the worm is necessary for morphological identification, except for some species with very distinctive pigmentation patterns (e.g., *Siphonenteron bilineatum*, *Lineus grubei*, *Kulikovia torquatus*). Specific identity of headless fragments can be determined using molecular barcoding (*see* **Note 3**).
7. If keeping track of the number of individuals, or maintaining individual identity is important, then use a single vial per collected individual. Nemerteans often fragment after collection, altering the original number and size of collected worms.
8. It might take several hours for worms to emerge, depending on how many of them were in the rubble, how much rubble is in each tray, air and water temperature.
9. As the rubble decays, water quality may degrade rapidly. There is a trade-off between waiting for a longer period to catch any straggler worms and allowing advancing putrefaction to generate a pungent foul smell.
10. It is highly advisable not to wait more than 24 h before disposing of the rubble, especially in warmer weather. When disposing of the rubble, return it as close to its source as possible, to avoid translocating organisms.
11. This is true for many species of nemerteans, though others might survive for only days or hours. Ability to survive for long periods without feeding is fundamental to survey regeneration capabilities: since anteriorly amputated worms usually lack a mouth to feed, they must be able to tolerate starvation long enough to regenerate a mouth. Specimens dying shortly (i.e., <2 days) after amputation lead to weaker inferences about an absence of anterior regeneration in a species. In contrast, beheaded specimens surviving for weeks or months without any signs of regeneration yield stronger evidence of absent anterior regeneration.
12. Nemertean cultures usually perform better when glass rather than plastic containers are used.
13. *Lineus sanguineus* individuals can tolerate a large range of densities, from single specimens to over a 100 worms in a single dish. Unless rearing resources are limited (i.e., limited bowls or shelf space), it is probably best not to keep more than 10–20 worms per culture dish. Although intraspecific aggression or cannibalism has not been reported, it is harder to keep track of a larger numbers of worms, as they tend to aggregate in dense "knots" from which it is quite difficult to isolate individual specimens.

14. Worms secrete a sticky mucus and will easily stick to the inside of the plastic pipette during transfers. To avoid this, try to use swift movements to aspire worms into the pipette and expel them into the container they are being transferred to. If worms stick to the inside of the pipette, try to energetically squeeze and release the bulb so that fast moving water dislodges them from their gooey grasp. If that fails, a last resort option is to cut open the pipette and rescue the worm with a paintbrush.
15. *L. sanguineus* individuals usually coil into a spiral (shorter animals) or knot (longer animals) when kept in vials. After being transferred to the glass bowls, worms should eventually begin to crawl around the bowl. Sometimes worms will curl themselves into a knot and secrete a mucus cocoon. If needed, the cocoon can be opened carefully with tweezers under a dissecting scope.
16. Some nemerteans tend to crawl upward, moving above the water level and sometimes lodging between the edge of the container and the covering lid. While they might survive for some time outside of the water in humid environments, they will eventually dry up and die. It is a good idea to check frequently after an initial culture is setup to see if this behavior shows up. Worms clinging on the container wall out of the water can be pushed back down by squirting them with water using a plastic pipette.
17. Keep an eye for evaporation or fouling of the water. Always make additional checks after feeding the worms (see Subheading 3.4), as food particles transferred to the culture dish along with recently fed worms can quickly spoil and foul the water. If a layer or biofilm of mucus and bacteria builds up over the surface of the bowl, move the worms to a new, clean bowl and add filtered sea water.
18. Laying out the liver homogenate in stripes before freezing facilitates feeding standardized amounts, and avoids freeze–thaw cycles by allowing thawing only the necessary amount of homogenate.
19. Many more worms can be placed into a single feeding dish, but as the number increases it becomes increasingly difficult to keep track of each individual's feeding performance and thus to ensure that all individuals are feeding adequately.
20. Allow worms time to detect the presence of food. Different individuals may take different time to do so, especially recently collected worms. Once they detect food, they will usually approach the liver piece and start eating. Worms in recently established cultures may initially not accept or eat the food being offered. A learning period involving several tries is sometimes required before they first try a new food item. Once they

successfully pass this period, they usually become much more eager to feed on the same substrate in subsequent occasions. However, if worms keep rejecting the food after several attempts, it might be worth trying with a different item.

21. If less manipulation of worms is desired, it is also possible to add the food directly to the culture dishes, and then remove uneaten food pieces. This however tends to accelerate fouling of water, so more care in changing sea water will be needed.
22. The number and size of fragments that can be obtained from a single individual varies with the original size of the specimen (i.e., long worms might yield over two dozen small fragments). In turn, the size of the fragments determines the size of the regenerated individuals.
23. Cold water will slow down worms enough to make a reasonable accurate cut. However, if highly precise amputations are needed, cold might not be enough to keep the worm in position. In those cases, animals can be anesthetized using <1.5% ethyl carbamate, <5% chloral hydrate, <1.5% chlorobutanol or 7.5% MgCl [17, 22]. To avoid passive displacement, the worm can also be placed over wet filter paper [23].
24. The size of the fragments can be as small or large as needed, but the probability of successful regeneration often decreases if a fragment's length is less than its width, or if it is made too close to the posterior end [10, 24]. If very small worms are desired, it might be necessary to make two successive rounds of amputation and regeneration.
25. Fragments that do not survive the amputation usually die within the next few hours. Dead or dying fragments stop all muscular and ciliary movements and usually start changing color and begin disintegrating.
26. *L. sanguineus* can live at a wide range of temperatures, if temperature does not change too abruptly. However, warmer temperatures (e.g. room temperature, ~24 °C) promote the growth of microorganisms and accelerate physiological processes of worms, which might be detrimental for cultures. Warmer temperatures, however, also accelerate developmental processes, including regeneration.
27. It is very common for regenerating fragments to secrete protective mucus and surround themselves in a spherical cocoon. They will complete regeneration within the cocoon, but if observation of the progress of the regeneration process is desired, can might be removed from the cocoon by carefully prying it open with a couple of sharp tweezers under a dissecting scope.
28. Change water sooner if microbial growth is observed.

29. Time to complete regeneration varies, depending on the size of the fragments, temperature, and condition and strain of the original individual, but usually by 2 weeks it should be possible to see ocelli, brains, mouth and a proboscis on the regenerate (Fig. 1c).

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