Journal of Visualized Experiments

Establishing an octopus ecosystem for biomedical and bioengineering research

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE62705R3
Full Title:	Establishing an octopus ecosystem for biomedical and bioengineering research
Corresponding Author:	Galit Pelled Michigan State University East lansing, Michigan UNITED STATES
Corresponding Author's Institution:	Michigan State University
Corresponding Author E-Mail:	pelledga@msu.edu
Order of Authors:	Tyler Wade VanBuren
	Carolina Cywiak
	Petra Telgkamp
	Christiane Mallett
	Galit Pelled
Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Bioengineering
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	East Lansing, Michigan, United States
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	
Please confirm that you have read and agree to the terms and conditions of the video release that applies below:	I agree to the Video Release

TITLE:

Establishing an Octopus Ecosystem for Biomedical and Bioengineering Research

AUTHORS:

Tyler VanBuren[1,2], Carolina Cywiak[1,2], Petra Telgkamp[1,2], Christiane L. Mallett[3,4], Galit Pelled[1,2,4*]

[1] Department of Biomedical Engineering, Michigan State University, East Lansing, MI, USA
[2]Neuroengineering division, The Institute for Quantitative Health Science and Engineering, Michigan State University, East Lansing, MI, USA
[3]Biomedical Imaging division, The Institute for Quantitative Health Science and Engineering, Michigan State University, East Lansing, MI, USA
[4]Department of Radiology, Michigan State University, East Lansing, MI, USA

Email Addresses of Co-authors:
Tyler VanBuren (vanbur28@msu.edu)
Carolina Cywiak (cywiakca@msu.edu)
Petra Telgkamp (telgkamp@msu.edu)
Christiane L. Mallett (cmallett@msu.edu)
Galit Pelled (pelledga@msu.edu)

Corresponding Author:
Galit Pelled (pelledga@msu.edu)

SUMMARY:

Understanding the unique physiological and anatomical structures of octopuses can greatly impact biomedical research. This guide demonstrates how to set-up and maintain a marine environment to accommodate this species and includes state-of-the-art imaging and analytical approaches to visualize octopus' nervous system anatomy and function.

ABSTRACT:

Many developments in biomedical research have been inspired by discovering anatomical and cellular mechanisms that support specific functions in different species. The octopus is one of these exceptional animals that has given scientists new insights into the fields of neuroscience, robotics, regenerative medicine, and prosthetics. The steps taken to begin research with this species of cephalopods require the set-up of complex facilities and intensive care for both the octopus and its ecosystem that is critical for the project's success. This system requires multiple mechanical and biological filtering systems to provide a safe and clean environment for the animal. Along with the control system, specialized routine maintenance and cleaning are required to effectively keep the facility operating long term. It is advised to provide an enriched environment to these intelligent animals by changing the tank's landscape, incorporating a variety of prey, and introducing challenging tasks for them to work through. Our results include MRI and a whole-body autofluorescence imaging as well as behavioral studies to better understand their nervous system. Octopuses possess unique physiology that can impact many

areas of biomedical research. Providing them with a sustainable ecosystem is the first crucial step in uncovering their distinct capabilities.

INTRODUCTION:

New concepts in biomedical research and biomedical engineering are often inspired by identifying specific strategies that biological species possess to address environmental and physiological conditions and challenges. For example, understanding the fluorescence properties in fireflies has led to the development of new fluorescent sensors that can report cellular activity in other model organisms[1]; identifying ion channels activated by light in algae has led to the development of cellular and temporal specific light-based-neuromodulation[2-5]; discovering proteins in glass catfish that navigate according to the Earth's magnetic field has led to the development of magnetic-based-neuromodulation[6-11]; understanding the siphon reflex in *Aplysia* has been instrumental to understanding the cellular basis of behavior[12-14].

Researchers continue to expand on the current bioengineering and phylogenetic toolbox by taking advantage of the unique strengths and novel perspectives on physiological functions that non-conventional lab species hold. Federal agencies are beginning to support these lines of studies by funding novel work performed on diverse species.

One genus of animals with unique anatomy and regeneration capabilities as well as the adaptive control of each of its arms, fascinating biologists and engineers, and captivating audiences from every part of the society is the *Octopus*[17]. Indeed, many aspects of octopus' physiology and behavior have been studied over the past decades[15-26]. However, recent development in molecular and evolutionary biology, robotics, motion recording, imaging, machine learning, and electrophysiology accelerate discoveries related to octopus physiology and behavior and translate them to innovative bioengineering strategies[27-39].

Here we describe how to set up and maintain octopus husbandry, which would be of interest and relevance to scientists and engineers from different backgrounds, scientific interests, and goals. Nevertheless, our results focus on the application of octopuses in neuroscience and neuroengineering research. The octopus has a highly developed nervous system with 45 million neurons in the central brain, 180 million neurons in the optic lobes, and additional 350 million neurons in the eight axial cords and peripheral ganglia; for comparison, a dog has a similar number of neurons and a cat only half of it[40]. Unlike the vertebrate nervous system, there are only 32K efferent and 140K afferent fibers connecting the millions of neurons in the octopus' brain to the millions of neurons in each of their arm's axial cords[40-42]. These relatively few interconnecting fibers suggest that most of the details for the execution of the motor programs are stored in the axial cord itself, emphasizing the unique distributed control the octopuses possess. The octopus's arms have extraordinary fine motor control enabling them manipulation skills such as opening jar lids, even when they are inside the container. This motor capability is specific to octopuses and other animals, in the class Cephalopod (cuttlefish and squid), do not have prehensile arms.

Indeed, through hundreds of millions of years of evolution, the octopus has developed a remarkable and sophisticated genome and physiological system[43,44] that have inspired the development and progress across scientific and engineering fields. For example, a water-resistant adhesive patch based on the octopus' suckers allow them to grip object and prey[45]; a synthetic camouflaging, octopus skin that can transform a flat, 2D surface to a three-dimensional one with bumps and pits[46]. Miniature soft and autonomous robots (i.e., Octobots) that in the future could serve as surgical tools inside the body[47]; and an arm (i.e., OctoArm) attached to a tank-like robot[48] have also been developed. Many species of octopuses are used in biomedical research e.g., *Octopus vulgaris*, *Octopus sinensis*, *Octopus variabilis*, and *Octopus bimaculoides* (*O. bimaculoides*); *O. vulgaris* and O. *bimaculoides* being the most common[34,49,50]. The recent sequencing of different octopus genomes makes this genus of particular interest and opens new frontiers in octopus research[34,43,51,52].

O. bimaculoides used in our set-up is a medium-sized species of octopus, first discovered in 1949, that can be found in shallow waters off the Northeast Pacific coast from central California to the South of Baja California peninsula[17]. It can be recognized by the false eyespots on its mantle below its eyes. Compared to Giant Pacific Octopus (*Enteroctopus dofleini*) *and* Common Octopus (*O. vulgaris*)*, the* California Two Spot (*O. bimaculoides*) is relatively small in size, starting out smaller than a few centimeters, growing fast as juveniles. When raised within a laboratory, the adult mantle size can grow to an average size of 100 cm and weigh up to 800 g[53,54]. Octopuses have a rapid growth period within their first 200 days; by then, they are considered adults and continue to grow throughout the rest of their life[55-57]. Octopuses can be cannibalistic, especially if housing both sexes together within a tank; therefore, they need to be housed individually in separate tanks[58].

PROTOCOL:

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Michigan State University.

1. Octopus tank equipment set-up

1.1. First, obtain all non-biological materials for an aquarium that will be incorporated into the marine environmental system, as shown in the **Table of Materials**. Sizes are provided in inches.

1.2. Wash all tubing, piping, and filter system parts prior to the installation with 70% ethanol and DI water. Do not use soap or any other chemicals when cleaning.

1.3. Place a fiberglass table 13 inch x 49 inch x 1 inch (Part #71) with four table legs made of carbon fiber and are 2 inch x 2 inch x 23 inch (Part #72). Attach the legs directly under the corners of the tabletop.

1.4. Below the top surface, between each of the table legs, attach 2 inch x 2 inch long (Part #72) carbon fiber stabilization braces to the underside of the table and directly against the edge of the top shelf. Attach with screws another same-sized shelf directly on the ground below the

table. Let the pump (see **Table of Materials**) sit directly on the bottom shelf surface while the tank sits on the top surface. This system is shown in **Figure 1**.

NOTE: Water output from the tank is gravity fed and all tubing, except the ones feeding in and out of the tank, need to be lower than the bottom of the tank to ensure maximum drainage head pressure.

[Place **Figure 1** here]

1.5. Drill a single 1 ¾ inch hole, 2 inches from one of the sides of the tank, using glass cutting drill bits. The bottom of the water output suction screen will determine the elevation of the output hole as shown on the right side of **Figure 2a**. The water level will be determined by the suction screen and will need to be at least 6 inches from the top of the tank allowing for a water splash zone.

1.6. Use a PVC primer and cement to permanently connect the sections. To do so, first, slide the end of the intended male PVC pipe into the end of the female pipe. Place a piece of painters' tape on the outside of the male part that is still visible to prevent the primer and the cement from showing on the outside of the pipe. Separate the parts after taping and place a light coat of primer on the outside of the male pipe following the application of the cement in the same area.

1.7. Refit the male pipe into the female pipe, as soon as possible, after the application of cement and remove the tape. 24 h after the application of the primer and cement, wash out newly connected parts with DI water. For curing time look at the cement product for further directions.

NOTE: Ensure the setup of all tubing and equipment is placed properly prior to using PVC primer and cement; pipe length requirements may vary.

1.8. Next, permanently connect the 1 inch outer diameter (OD) end of the suction screen to the 1 inch inner diameter (ID) end of the elbow joint. Connect the end of the elbow joint to straight PVC tubing (1 inch OD). Connect the other side of the straight tubing then to the 1 inch ID of the through-wall straight adapter female socket connect.

NOTE: ID refers to the widest distance between the inside walls of the pipe. OD refers to the outside of the tubing with.

1.9. Permanently connect the through-wall straight adapter to a straight 4 inch long PVC pipe with a 1 inch OD (from step 1.8). This pipe will face out of the tank.

1.10. Permanently connect the straight pipe to the center of the PVC connector (1 inch ID Tee shaped; from step 1.9). Next, permanently connect two 6 inch long (Part #69) pipes (1 inch OD) to both the opposite ends of the tee connector—one facing directly up for the air release and the other directly down for water flow.

1.11. Permanently connect the downward extended straight pipe (from step 1.10) to a female socket barbed pipe (1-inch ID) straight adaptor. Attach a 36 inches long rubber tube (¾ inch ID) to the barbed pipe adaptor.

1.12. Place the cooling system between the water output tubing and the sump system.

1.13. Attach the ¾ inch barb fittings, that comes with the system, to the chiller unit's input and output ports. Put the rubber tubing (from step 1.11) on the inlet fitting of the chiller.

1.14. Connect a new piece of ¾ inch ID tubing (from step 1.13) from the chiller output (from step 1.12) to the inlet of the sump system as shown in **Figure 2b**.

1.15. Next, place the 4 inch x 12 inch sock filter, with pore size of 200 µm, into its designated area as shown in **Figure 2**. Also, as depicted in **Figure 2**, place the protein skimmer and the return pump into their appropriate areas. Along with the return pump, attach the automatic top off float valve to the inside wall of the pump area, 2 inches above the top of the pump's water inlet; do not block the pump from being removed from the tank, if needed.

1.16. Permanently connect a straight 12-inch-long tube (¾ inch OD) to the pump's outlet (from step 1.15). On the other end of the ¾ inch OD straight tube, permanently connect the tube's OD to a ¾ inch ID 45° elbow joint. To the other end of the joint, permanently connect a ¾ inch OD tubing.

1.17. Attach the other end of the straight tube (from step 1.16) to the 3/4 inch ID of a straight reducing adaptor. Permanently connect the larger adapter end (2-inch OD) to the input of the UV light.

NOTE: Straight tubing lengths may vary.

1.18. Next, match the placement of UV light inlet with pump's output pipe (from step 1.17) so that the pipe is not bending between light and pump (from step 1.15). Drill holes into the stabilization brace to match the UV light attachment holes. Match the size of screws with the drill bit and attach the UV light to the table using the screws given.

1.19. Permanently connect the 2-inch side of another reducing adaptor to the output of the UV light (from step 1.18). Attach a 1-inch OD of a 5-inch long straight tube to the adaptor's 1-inch ID. Next, connect a 90˚ corner piece with the 1-inch ID to the 1-inch OD tube; have the unattached end of the corner piece pointing toward the side of the tank where the water input is intended to go (same side as in step 1.5).

1.20. Permanently connect the other end of the corner (from step 1.19) to a 6 inches long tube (Part #69) having 1-inch OD with the input of the flow control unit (Part #2). Permanently connect

another 1-inch OD tube (Part #69) to the output of the flow monitoring unit, which length must extend at least 3 inches beyond the side of the tank.

1.21. Using a ¾ inch glass cutting drill bit (Part #1), cut a new hole 3 inches above the intended waterline and 2 inches away from the side of the tank (**Figure 1a**) on the side opposite to the one having water output hole. Attach another through-wall bulkhead fitting with a 1-inch slip (Part #77) facing out of the tank.

1.22. To the bulkhead slip connect a straight tube with the 1-inch OD and 4 inches length (Part #69) permanently. Cut down the tubing from the last part of step 1.21 to match the distance this tubing extends from the tank. Permanently connect a 90° tube (Part #65) to each of the open pipes and cut a final 1-inch OD straight tube (Part #69) that permanently connects both corner pieces.

NOTE: **Figure 3** shows a simple representation of the aquarium system.

1.23. Setup the rest of the control system (Part #34), first mounting the power strip (Part #53) to the table itself or to a nearby wall. Next to it mount the fluid monitoring module (Part #2).

1.24. Connect the flow sensor, power strip, and the leak detection sensors to the module. Set up the growth light (part #26) that is attached to the algae bin (**Figure 2**).

1.25. Plug in the flow sensor, UV light, growth light, pump, and protein skimmer to the energy bar. Setup the water control system programming according to the manufacturer's manual.

1.26. Prepare saltwater by mixing half a cup of commercially available salt mix with 1 gallon of reverse osmosis (RO) or deionized (DI) water. Make 45 gallons to fully fill one tank and sump system.

1.27. Turn on the pump within the sump system flow controller and keep adding saltwater until the automatic top off valve is in the off position so no additional freshwater is required.

1.28. Once the water is full, stop filling and turn on the water chilling unit to set the temperature between 18 ˚C to 22 ˚C as this is the preferable temperature range[53]. Turn on the protein skimmer.

1.29. Add 30 kg of crushed coral to the bottom of the tank as well as a layer of crushed coral to the bottom of the algae bin. Add in multiple live rocks and any other additions to the octopus environment. Place a top to cover the opening of the tank.

NOTE: Live rocks are dead coral that are inhabited by macroscopic marine life such as bacteria and algae.

1.30. Add nitrifying bacteria used in the saltwater aquariums as directed on the packaging. Keep adding this as directed, checking temperature, salinity, pH, ammonia, nitrite, and nitrate daily with water testing kits, pH sensor, and temperature sensor. Safe values for ammonia, nitrite, and nitrate levels are below 0.5 ppm, 0.25 ppm, and 10 ppm respectively[58].

1.31. Ensure UV light is turned off for the days nitrifying bacteria is being added to allow for the saltwater microorganisms to grow. After parameters are within safe ranges, the UV light can be reactivated.

1.32. After the system is established, also check that the pH and oxygenation is at 8.0–8.4 and $4.5 \pm 0.95 \ \frac{mgO_2}{L}$ [59], respectively. Prior to adding any animals to the aquarium, check for the presence of any copper and oxygen levels within the system using a copper water testing kit.

NOTE: Copper causes damage to invertebrates and it interferes with osmoregulation in fish gills[60,61].

1.33. If copper is found in water, test the DI/RO water source. After determining that the water source does not contain copper, perform a 30% water change and place the activated carbon block (Part #46) within water. If the problem persists, perform a full water change and clean all the parts.

1.34. After all the water parameters are determined to be within safe levels, add 10 ghost shrimps into the system at least a week prior to adding the octopuses. This will help introduce biomass for bacteria and indicate the overall water quality.

1.35. Add additional aquarium ecosystem inhabitance to the algae bin. This includes *Chaetomorpha* spp. (spaghetti algae), *Trochus Sp.* (banded trochus snail), and *Mercenaria mercenaria* (cherrystone clams).

[Place **Figure 2** here]

[Place **Figure 3** here]

2. Storage tanks

2.1. Set up two tall 60-gallon water storage tanks, one for the saltwater and the other for RO water. Ensure that the freshwater tank's maximum fill line is taller than the table. Attach a ¼ inch tubing to the automatic top of the float valve in the sump system and attach the other end of tubing to the bottom of the freshwater tank.

NOTE: This is to refill if water evaporates. Salt will stay in the water.

2.2. Fill the saltwater tank with water and add the proportional amount of salt to the tank. Continuously aerate the saltwater storage tank for mixing and proper oxygenation. Wait for an hour before use to ensure full mixing of the salt.

NOTE: The saltwater tank is useful for refilling the tanks after cleaning.

3. Food tank setup

3.1. For keeping shrimp alive for longer than a week, store them in a separate tank from the octopus with the salinity below 30 ppt and the temperature close to 25 ˚C.

3.2. To do so, one week after octopus tanks are matured, transfer 8 gallons of matured saltwater to the shrimp tank. Add 15 kg of crushed coral to the bottom of the tank. Add a few live rocks to the tank for hiding spots for molting (**Figure 4**).

NOTE: Matured seawater refers to the process of allowing marine bacteria to grow within the saltwater as shown in step 1.30.

3.3. Attach a cannister filter to the edge of the tank. Setup the cannister filter as directed by the manufacturer. Add an air pump next to the tank connected to a tube with an attached air stone put into the tank.

3.4. Weekly, clean the filter and change the filter pads every week. Also, 25% of the water will need to be changed at the same time. Check nitrogen, pH, and temperature parameters daily in the food tanks with water testing kits. If water nitrogen parameters remain high, additional water changes will be needed and add a nitrogen absorbent bag; or if problems persist longer than a month, the shrimp will need to be moved to a larger tank.

3.5. Add shrimp as soon as crushed coral sediment is dissipated. To add shrimp first, on arrival, move the shrimp without shipping water to the small intermediatory saltwater tank for 5 min to remove biowaste. Then, the shrimp can be added directly to the tank. Mosquito fish, on arrival, can be added directly to the shrimp tank.

NOTE: Shrimp and Mosquito fish can be purchased from any live animal commercial supplier on material sheet or other food suppliers. It is also possible to offer octopuses defrosted shrimp.

3.6. Feed shrimp and fish with fish flakes, dead vegetation, or algae[62], as directed on food instructions.

3.7. For the crab tank, add 1 gallon of saltwater and 10 kg of pebbles. Pile the pebbles on one side leaving dry land on one side and fill the other with water. The optimal environmental water parameters for these invertebrates should be 30–35 ppt and 22–25 ˚C for salinity and temperature[11,63], respectively.

3.8. Add fiddler crabs directly into the tank (**Figure 4)**. Crabs will spend most of their lives on land but can be underwater for a few days at a time, making the tank that is partially underwater crucial for their long-term survival.

3.9. Feed fiddler crabs once per day by adding fish flakes into the dish on the dry area of the tank. Clean weekly by removing crabs and changing 100% of the saltwater. Cleaning the pebbles.

3.10. Store marine bivalve mollusks (clams and mussels) within the saltwater tanks for the octopuses to open themselves and provide another water filtering mechanism[64].

3.11. Place mussels inside a separate unoccupied tank for the first week to avoid placing an unnecessary waste load on octopus tank's filtering system.

NOTE: While the mussels have been the octopus' food of choice, they are more likely to die soon after arrival and will substantially increase the biological waste within the tank if they are present in large quantities.

4. Introduction of octopus to the tank

4.1. Ensure ammonias, nitrite, and nitrate levels are below 0.5 ppm, 0.25 ppm, and 10 ppm respectively. Have water hand pump available to remove octopus ink from the tank. It is also recommended to have two people for this procedure.

4.2. On arrival, place the bag on the scale and subtract the weight of the bag after the octopus is removed. Add an air stone to the bag to increase the water oxygenation while transferring the animal to their tank. Measure the shipping water's temperature and salinity. Record cases of prolonged illness after shipment.

4.2.1. Without transferring any water from the bag to the tank, hang the transport bag over the corner of the tank with the bag partially submerged in the tank water to begin changing the temperature of the transportation bag. Remove 10% of the water from the bag and dump down the sink. Add the same amount of water from the tank to the bag. Repeat every 10 min until the water temperature in the bag is no more than 1° different than the water temperature in the tank.

4.2.2. Once the temperature difference of the bag and the tank are within 1°, ensure gloves are worn to move the octopuses to their individual tank. To move, place both hands under the octopus to provide support during the transfer; the second person will need to gently pull the suctioned arms from the side of the bag.

4.2.3. Once the octopus is out of the bag, move it quickly into the water of its new habitat transferring as little water from the shipping bag as possible. Use the hand pump to remove any ink the octopus releases when in the tank. Now weigh the bag with water to obtain approximate weight of the animal.

4.3. For the first 2 weeks after arrival, monitor the octopus' daily consumption that should be around 4% to 8% of their weight[58,65,66]. The octopus should be checked on four times a day; this can be decreased to twice per day after 2 weeks. Weigh every two weeks to adjust their food consumption as needed.

NOTE: Some species of octopus are known to escape from their tank, so it is advisable to place a 2.5 kg weight on the lid of their tank.

5. Daily care

5.1. Using a commercially available saltwater testing kit for pH, ammonia, nitrite, and nitrate, add the kit-directed amount of tank water to the four test tubes provided with the kit. As specified on the testing kit, add the amount of colorimetric reactant to the corresponding tube.

5.2. If ammonia, nitrite, and nitrate levels are above 0.5 ppm, 0.25 ppm, and 10 ppm respectively, wash the biomass out of the sock filter or change to a new sock filter. Additionally, clean out biomass from the top of the skimmer with a brush and add additional denitrifying bacteria to the tank. If problems persist, then replace 25% of fresh saltwater.

NOTE: The above steps reduce nitrogen compounds within the ecosystem.

5.3. Remove all dead crab and shrimp carcasses from the tank as well as any octopus fecal matter using a hand pump. Remove all the remaining living crabs from the tank and move them back to the storage tank. Next, rearrange large objects within the tank.

5.4. Next, introduce half the number of the crabs that the octopus would eat daily to the tank weighing 1.25 +/- 0.25 g. Feed defrosted shrimp or small male fiddler crabs to juvenile octopuses. Depending on the experiment, crabs and shrimp can be introduced anywhere in the tank or to the octopus directly.

NOTE: Octopuses food consumption is 4%–8% of their weight daily[67]. Frozen shrimp can also be provided as a food source based on the octopus' weight.

5.5. Offer five ghost shrimp daily. On an average, three were consumed in this experiment. To provide a variety of food to the octopus, give one live clam or mussel once a week and always maintain three mosquito fish inside the tank.

NOTE: Giving the animals a variety of food is not required and can prevent animals from being enticed by food during experiments. The feeding schedule used here to best monitor octopus feeding and behavior is to introduce half the number of crabs based on weight and increase the number of shrimp to five in the morning. In the evening, introduce the second half of the crabs to the tank.

6. Weekly sanitation

6.1. Shutdown the skimmer, pump, and algae bin lights prior to cleaning the sump system. Then, turn off the automatic valve of the system prior to removing water. Finally, remove the skimmer and all the water only from the sump system.

6.2. Lightly scrub algae bin to remove most of the biomass from its walls. Clean the rest of the sump area with a brush. Remove the sock filter, clean out with vinegar, and let it dry; rotate with another sock filter each week replacing with new ones every three months. Remove and clean out biomass from the top of the skimmer weekly.

NOTE: Avoid using metal to clean the plastic as it will create scratches that could be prone to microbial growth.

6.3. Put the skimmer back into the system and begin refilling with saltwater. When the pump area is beginning to fill, all the systems can be turned back on. Stop adding water when automatic top of the float valve is in the off position.

[Place **Figure 4** here]

[Place **Figure 5** here]

7. Care of unwell animals

7.1. Follow the guide reference[66] to assess octopus wellness.

NOTE: For female octopuses, end of life cycle normally begins after laying eggs. The animal will begin to decrease food consumption followed by stopping to eating altogether and become more lethargic. Lifespan after the end-of-life process varies. No further action can be taken except feeding and monitoring the animal. Senescent males will decrease food consumption and become lethargic[68].

8. Octopus anesthesia

8.1. Perform octopus anesthesia as detailed in Butler-Struben et al.[69].

8.2. Obtain a 6 L container with lid that is at least 15 cm tall. Place 4 L of water directly from the octopus's tank into the container and provide aeration for 4 L of saltwater using a small air pump with air stone to disseminate oxygen to the water environment[58].

8.3. Prior to the octopus introduction, add 1% EtOH to the container. Before handling the octopus, record the number of breaths per minute by counting the exhalation of water from the syphon.

NOTE: For octopuses within researcher's laboratory, the baseline respirations is 16 – 24 breaths per minute.

8.4. Prior to moving the octopus, record the octopus' skin pigmentation and baseline breathing rate. Remove the octopus from the tank using a clean 4 L open mouth container by scooping it up with its surrounding water.

NOTE: During anesthesia, breathing rates do not necessarily indicate complete anesthesia.

8.5. Weigh the octopus while in the container, and then move it by placing both hands around the octopus' body and lifting it up. A second person may be needed to remove the suctioned limbs from the container walls.

8.6. Quickly move the octopus into the prepared container with 1% EtOH. Close the lid to prevent a possible escape.

8.7. Record the respiration of the octopus per minute by counting the exhalation of water from syphon at the end of the first 5 min. If the respiration remains above baseline and the animal continues to respond to a light pinch, add an additional 0.25% EtOH to water. The addition of ethanol to water can continue to a maximum of 3% EtOH.

NOTE: One indication that the octopus is unconscious is its loss of control of its chromatophores. In this case the skin appears paler than normal. A further indication is to lightly pinch the arms and test whether there is a motor response. If there is still no response at this point, the octopus is unconscious, and experiments can be performed.

8.8. While under anesthesia monitor the octopus' breathing and color to ensure it remains unconscious for the duration of the procedure. If the octopus begins to awaken during the procedure, add an additional 0.25% EtOH.

8.9. For reversing the effects of ethanol anesthesia, transfer the octopus to a new 4 L or greater tank of oxygenated water from its permanent holding tank. Once the respirations return to normal, the octopus becomes active, and its skin returns to normal pigments; it can be moved back to its tank.

9. Octopus euthanasia

9.1. Follow the international standards for octopus euthanasia as detailed in Fiorito et al., Moltschaniwskyj et al., and Butler-Struben et al[57,58,69].

9.2. Prepare a new 6 L container with 4 L of water from the octopus' holding tank. Mix in $MgCl_2$ to a concentration of 4% to the euthanasia tank. Perform steps from 8.1 to 8.9 to anesthetize the octopus.

9.3. Move the octopus to the euthanasia tank. After the breathing stops, wait for 5 min and perform a decerebration of the octopus or keep in the euthanasia tank for 5 additional minutes.

10. Behavior of *O. bimaculoides*

10.1. Do not feed the octopus on the mornings when they will be trained to use a screwcap container. Setup a camera recording device pointing at the area intended for feeding.

10.2. Obtain a screwcap 50 mL tube with 1 mm diameter holes throughout the surface and the cap for water flow throughout the container. Place fiddler crab within the container. Place the weight within the container or attached to the outside for it to remain at the bottom of the tank.

10.3. Place a container at bottom of the tank within the open area and in sight of the octopus and the camera. If the crab has not been eaten after 4 h, then remove it from the tube and resume the feeding schedule for the day. Keep performing this exercise daily.

NOTE: This is shown in **Figure 6** and discussed in the representative result section.

11. Octopus MRI

NOTE: Previously, evoked functional MRI responses in the octopus's retina were measured in anesthetized animals[70]. Here, we obtained an ultra-high spatial resolution MRI of the octopus' nervous system that required hours of scanning. Thus, this was performed in a euthanized *O. bimaculoides*.

11.1. Obtain MRI images using a 7T system. Wrap the octopus in a kitchen-grade polyvinyl chloride plastic wrap to maintain the hydration of tissue. Place the octopus on the wrap, tuck in the ends, and then roll to seal.

11.2. Use a volume transmit/receive coil with a 4 cm diameter to acquire images of the brain and multiple arms. Use T1 weighted RARE sequence with the following parameters: Repetition time (TR) of 1500 ms, echo time (TE) of 20 ms, 117 x 117 x 500 µm resolution, 100 averages, RARE factor 8. These are typical MRI parameters for imaging rodent brains. Using a RARE factor makes the imaging faster, while 100 images are averaged together to increase the signal-to-noise ratio[71].

11.3. Image the octopus arm using an 86 mm volume transmit coil and a 4 x 4 cm 4-channel array receive coil. Cut off an arm using surgical scissors and place it in a 15 mL conical tube filled with phosphate-buffered saline.

NOTE: The sequence was a T1_weighted inversion recovery sequence (MP-RAGE) with parameters: TR/TE = 4000/2.17 ms, inversion delay 1050 ms, 100 x 100 x 500 µm resolution, 9 averages, scan time 1.5 h (**Figure 7**). An inversion-recovery sequence nulls the signal from water and increases contrast within the image; this sequence was chosen because it allows visualization of the internal anatomy of the arm[72].

12. Cryo-fluorescence tomography (CFT) imaging

12.1. Flash-freeze the octopus: Work in a fume hood. Cover the bottom of a Dewar with dry ice, and then fill with hexanes. Slowly lower the octopus into the hexanes over about 10 min, adding fresh hexanes and dry ice as required to fully cover the octopus with cold hexanes. Keep the octopus frozen at -20 °C until it is embedded.

12.2. Embed and section the octopus: Create a rectangular mold of the appropriate size to hold the octopus using the tools provided by the CFT manufacturer. Cover the bottom of the mold with OCT (optimal cutting temperature) media (standard material used in histology laboratories) and let it freeze to a semi-solid gel.

12.3. Place the frozen octopus into the gel layer of the OCT, and then cover slowly with OCT in 2–3 layers. Between pouring steps, freeze the block steps until the OCT is at the gel stage. After the octopus is entirely covered, freeze the block for at least 12 h at -20 °C.

12.4. Load the sample into the cryo-fluorescence tomography system[73].

12.5. Section and image the entire euthanized O. bimaculoides at mesoscopic resolution using 3 emission/excitation filters thereby producing several 3D isotropic datasets.

12.6. When the sectioning reaches the arm and digestive system, transfer the sections to the slides for further histology.

12.7. Load the raw dataset into the reconstruction software from the CFT vendor specifically designed to enable fast processing.

12.8. Reconstruct a 3-dimensional stack using landmark alignment, histogram balancing, and fluorescence corrections and normalization, including the removal of subsurface fluorescence effects for each wavelength.

12.9. Once the final 3D stack is produced by the reconstruction tool, visualize the data with the imaging software tool and create fly-throughs with white light and fluorescence overlays along with 3D Maximum intensity projections (3D-MIPS), e.g., **Figure 8**[73].

REPRESENTATIVE RESULTS:

All the animals in our studies were obtained from the wild, and thus their exact age could not be determined and their stay in the lab was variable. Octopus condition was observed daily. We did not see parasites, bacteria, skin damage, or abnormal behavior. The average weight of animals was 170.38 +/- 77.25 g. Each animal inhabited their own 40-gallon. The mean ± standard deviation for the parameters recorded for a tank over a week were: pH 8.4 ± 0.0, salinity 34.06 ± 0.61 ppt, temperature 18.7 ± 0.75 °C, ammonia 0.11 ± 0.14 ppm, nitrite 0.25 ± 0.14 ppm, and nitrate 1.43 ± 2.44 ppm.

Behavior of *O. bimaculoides*: To understand the sensorimotor function as well as learning and memory capabilities of octopuses, unscrewing test tubes has been shown to be a useful test (**Figure 6**). It also provides an enriched environment that has been shown useful to maintain critical physiological mechanisms associated with neural degradation[74]. This test was performed daily with three octopuses, and it took the octopuses 4 days on an average to learn how to open a test tube.

[Place **Figure 6** here]

MRI of the octopus' nervous system: An MRI provides a means to visualize soft tissue with great spatial resolution. We acquired ultra-high spatial resolution images (100 microns voxels) of the *O. bimaculoides's* nervous system (**Figure 7**). This technique will allow to obtain detailed morphology and fiber tracking and orientation in a whole-animal preparation.

[Place **Figure 7** here]

Cryo-fluorescence tomography (CFT) imaging: The CFT is a state-of-the-art method that enables acquiring high-resolution imaging in a whole animal preparation. The system used only autofluorescence to generate 3-dimensional morphological image of the entire animal. As shown in **Figure 8**, this allowed to visualize the brain and the suckers that are positioned along the arm in the 470 wavelength (green) and the digestive system in the 555 (blue) and 640 (yellow) wavelengths.

[Place **Figure 8** here]

FIGURE LEGENDS:

Figure 1: Octopus tank setup. Water inlet and outlet (**a**). Three octopus tanks each with an area of 1.22 m x 0.3 m (**b**).

Figure 2: Sump system. Side view of the sump system (**a**). Top view of the sump system (**b**).

Figure 3: Aquarium with sump filtering system below the tank and environmental control units. Green arrows indicate direction of water flow through the system. Water flowing from section one to two for cooling and onto three to separate heavy biological matter from lighter matter. Heavy waste floats to the bottom and out to section five while the smaller biological matter flows into the sock filter within section four. Water flows from four under section five entering the protein skimmer in six to remove remaining waste within the water. Algae bin contains microorganisms to break down waste, ammonia, and nitrates as well as oxygenate the water. In the last part of the system, more water is added to account for evaporation prior to being pumped back into the tank.

Figure 4: Tank for fiddler crabs (*Minuca pugnax*). The bottom of the tank is half designated for dry bed and the other half for 2 cm of shallow saltwater.

Figure 5: Tank for ghost shrimp (*Palaemonetes paludosus*). Rocks in the shrimp tank provide places for the shrimp to hide and molt as well as for the growth of microorganisms.

Figure 6: Progression of an octopus unscrewing the lid of a tube. Use cameras for recording videos of green detection boxes generated from the camera software. In the last frame of the video blue object is the cap of the tube rising toward the surface of the tank after being removed by the octopus. Scale bar = 30 mm.

Figure 7: MRI of the octopus' nervous system. High resolution MRI characterization of the *O. bimaculoides* nervous system. We acquired *ex vivo* MRI images of the brain and the arms of the octopus that together form a nervous system that contains over 500 million neurons. The brain is in the center, and the two optic lobes are connected on each side (**a**). A coronal view of the arms. The axial cord can be seen in each of the seven arms captured in this view (**b**). A sagittal view of the suckers demonstrates a complex peripheral nervous structure (**c**). Scale bar = 5 mm.

Figure 8: Cryo-fluorescence tomography (CFT) imaging of *O. bimaculoides*. The entire octopus was embedded in a block and serially sliced while collecting white light and fluorescence images after each section. This produced a 3D isotropic data set with three fluorescence wavelengths. Scale bar = 30 mm.

DISCUSSION:

System Setup:

The aquarium ecosystem has been developed in a way that both mechanical and biological methods of filtering and oxygenating the water are employed. The filtering elements of the system utilizes sock filters, protein skimmers, and regular cleaning to maintain nitrogen and oxygen levels. More importantly, we also rely on marine microorganisms to consume the dangerous nitrogenous compounds and other biological waste as well as aerate the water through processes of photosynthesis. Additional methods, besides the use of algae, to add oxygen to the water is through exterior aerator with attached air stone. Prior to adding any bacteria, it is recommended to add live sand or crushed coral as a growth media. Without media the organisms will take longer to establish themselves within the system. This development will take 1–3 weeks to effectively breakdown biowaste and stabilize the nitrogen cycle within appropriate parameters.

Environmental Enrichment:

Cognitive and sensorimotor enrichment can assist in neurogenesis and the overall well-being of the octopus[75]. Enrichment can consist of sandy substrate, shells, rocks, and other structures that provide hiding places and cover. We often change the configuration of the structures within the octopus' tank and introduce new toys with interesting mechanics to motivate the octopus to explore. We found that it is best to use flowerpots with a hole at the bottom to house octopuses. This allows for less traumatic handling, where in a house with one entrance, the octopus may be

harmed when trying to be removed. The octopus enjoys interacting with large Legos and unscrewing jars with food placed within, as also described in Fiorito et al.[58]. Environmental enrichment is important for the octopus' cognitive and physiological health, which has been shown to impact critical regeneration mechanisms in the octopus' nervous system[74,75].

Improvements:

The setup of the system can be modified such as increasing the size of the tanks, using different sump systems, as well as different equipment. Further improvements that could be made are to add the cooling system after the sump pump output due to flow limitations caused by the cooling system. Additional improvements would be to introduce different types of algae to control nitrate levels as well as other prays, such as other non-poisonous mollusks and decapods, which the octopus may prefer as additional options.

Octopuses require constant care and attention and the methods employed within this protocol have proven to provide a stable and healthy environment for its inhabitants. While the methods outlined here are for *O. bimaculoides*, the basic aquarium setup can be employed for most marine animals with minor variations in the size of the system and equipment. These animals' unique characteristics make them ideal for many areas of research and the success of projects involving these animals depends on the diligence of the husbandry team. Octopuses with their incomparable abilities make them a remarkable and important animal model to employ in biomedical research.

ACKNOWLEDGMENTS:

This work was supported by NIH UF1NS115817 (G.P.). G.P. is partially supported by NIH grants R01NS072171 and R01NS098231. We would like to thank Patrick Zakrzewki and Mohammed Farhoud from Emit Imaging for the help and support in collecting and visualizing the data on the Xerra Imaging Platform. MSU has a research agreement with Bruker Biospin.

DISCLOSURES:

All the authors declare no conflicts of interest.

REFERENCES:

1 Wet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R., Subramani, S. Firefly luciferase gene: structure and expression in mammalian cells. *Molecular and Cellular Biology.* **7** (2), 725-737 (1987).

2 Han, X., Boyden, E. S. Multiple-color optical activation, silencing, and desynchronization of neural activity, with single-spike temporal resolution. *PLoS One.* **2** (3), e299 (2007).

3 Zhang, F. et al. Multimodal fast optical interrogation of neural circuitry. *Nature.* **446** (7136), 633-639 (2007).

4 Li, N. et al. Optogenetic-guided cortical plasticity after nerve injury. *Proceedings of the National Academy of Sciences of the United States of America.* **108** (21), 8838-8843 (2011).

5 Airan, R. D., Li, N., Gilad, A. A., Pelled, G. Genetic tools to manipulate MRI contrast. *NMR Biomedicine.* **26** (7), 803-809 (2013).

6 Cywiak, C. et al. Non-invasive neuromodulation using rTMS and the electromagnetic-perceptive gene (EPG) facilitates plasticity after nerve injury. *Brain Stimulation.* **13** (6), 1774-1783 (2020).
7 Hwang, J. et al. Regulation of Electromagnetic Perceptive Gene Using Ferromagnetic Particles for the External Control of Calcium Ion Transport. *Biomolecules.* **10** (2) (2020).
8 Lu, H. et al. Transcranial magnetic stimulation facilitates neurorehabilitation after pediatric traumatic brain injury. *Scientific Reports.* **5**, 14769 (2015).
9 Krishnan, V. et al. Wireless control of cellular function by activation of a novel protein responsive to electromagnetic fields. *Bioscience Reports.* **8** (1), 8764 (2018).
10 Mitra, S., Barnaba, C., Schmidt, J., Pelled, G., Gilad, A. A. Functional characterization of an electromagnetic perceptive protein. *bioRxiv.* 329946, (2020).
11 Hunt, R. D. et al. Swimming direction of the glass catfish is responsive to magnetic stimulation. *PLoS One.* **16** (3), e0248141 (2021).
12 Kandel, E. R., Krasne, F. B., Strumwasser, F., Truman, J. W. Cellular mechanisms in the selection and modulation of behavior. *Neurosciences Research Program bulletin.* **17** (4), 521-710 (1979).
13 Carew, T. J., Castellucci, V. F., Kandel, E. R. An analysis of dishabituation and sensitization of the gill-withdrawal reflex in Aplysia. *International Journal of Neuroscience.* **2** (2), 79-98 (1971).
14 Kandel, E. R. The molecular biology of memory storage: a dialog between genes and synapses. *Bioscience Reports.* **21** (5), 565-611 (2001).
15 Forsythe, J. W., Hanlon, R. T. Effect of temperature on laboratory growth, reproduction and life-span of octopus-bimaculoides. *Marine Biology.* **98** (3), 369-379 (1988).
16 Forsythe, J. W., Hanlon, R. T. Behavior, body patterning and reproductive-biology of octopus-bimaculoides from California. *Malacologia.* **29** (1), 41-55 (1988).
17 G. Pickford, B. M. The Octopus bimaculatus problem: A study in sibling species. *Bulletin of the Bingham Oceanographic Collection.* **12**, 1-66 (1949).
18 German Sumbre, Y. G., Graziano Fiorito, Tamar Flash, B. H. Control of octopus arm extension by a peripheral motor program. *Science.* **293** (5536), 1845-1848 (2001).
19 Gutfreund, Y. et al. Organization of octopus arm movements: a model system for studying the control of flexible arms. *Journal of Neuroscience.* **16** (22), 7297-7307 (1996).
20 Gutfreund, Y., Matzner, H., Flash, T., Hochner, B. Patterns of motor activity in the isolated nerve cord of the octopus arm. *The Biological Bulletin.* **211** (3), 212-222 (2006).
21 Hague, T., Florini, M., Andrews, P. L. R. Preliminary in vitro functional evidence for reflex responses to noxious stimuli in the arms of Octopus vulgaris. *Journal of Experimental Marine Biology and Ecology.* **447**, 100-105 (2013).
22 Hochner, B., Brown, E. R., Langella, M., Shomrat, T., Fiorito, G. A learning and memory area in the octopus brain manifests a vertebrate-like long-term potentiation. *Journal of Neurophysiology.* **90** (5), 3547-3554 (2003).
23 Hochner, B., Glanzman, D. L. Evolution of highly diverse forms of behavior in molluscs. *Current Biology.* **26** (20), R965-R971 (2016).

24 Hvorecny, L. M. et al. Octopuses (Octopus bimaculoides) and cuttlefishes (Sepia pharaonis, S. officinalis) can conditionally discriminate. *Animal Cognition.* **10** (4), 449-459 (2007).

25 Kier, W. M., Stella, M. P. The arrangement and function of octopus arm musculature and connective tissue. *Journal of Morphology.* **268** (10), 831-843 (2007).

26 Levy, G., Hochner, B. Embodied organization of octopus vulgaris morphology, vision, and locomotion. *Frontiers in Physiology.* **8**, 164 (2017).

27 Giorgio-Serchi, F., Arienti, A., Laschi, C. Underwater soft-bodied pulsed-jet thrusters: Actuator modeling and performance profiling. *The International Journal of Robotics Research.* **35** (11), 1308-1329 (2016).

28 Han, S., Kim, T., Kim, D., Park, Y., Jo, S. Use of deep learning for characterization of microfluidic soft sensors. *IEEE Robotics and Automation Letters.* **3** (2), 873-880 (2018).

29 Hanassy, S., Botvinnik, A., Flash, T., Hochner, B. Stereotypical reaching movements of the octopus involve both bend propagation and arm elongation. *Bioinspiration and Biomimetics.* **10** (3), 035001, (2015).

30 Hochner, B., Shomrat, T., Fiorito, G. The octopus: a model for a comparative analysis of the evolution of learning and memory mechanisms. *The Biological Bulletin.* **210** (3), 308-317 (2006).

31 Imperadore, P., Fiorito, G. Cephalopod tissue regeneration: consolidating over a century of knowledge. *Frontiers in Physiology.* **9**, 593 (2018).

32 Imperadore, P. et al. Nerve regeneration in the cephalopod mollusc Octopus vulgaris: label-free multiphoton microscopy as a tool for investigation. *Journal of the Royal Society, Interface.* **15** (141), 20170889 (2018).

33 Levy, G., Flash, T., Hochner, B. Arm coordination in octopus crawling involves unique motor control strategies. *Current Biology.* **25** (9), 1195-1200 (2015).

34 Li, F. et al. Chromosome-level genome assembly of the East Asian common octopus (Octopus sinensis) using PacBio sequencing and Hi-C technology. *Molecular Ecology Resources.* **20** (6), 1572-1582 (2020).

35 Lopes, V. M., Rosa, R., Costa, P. R. Presence and persistence of the amnesic shellfish poisoning toxin, domoic acid, in octopus and cuttlefish brains. *Marine Environmental Research.* **133**, 45-48 (2018).

36 Mazzolai, B., Margheri, L., Dario, P., Laschi, C. Measurements of octopus arm elongation: Evidence of differences by body size and gender. *Journal of Experimental Marine Biology and Ecology.* **447**, 160-164 (2013).

37 McMahan, W. et al. in *Proceedings 2006 IEEE International Conference on Robotics and Automation, 2006. ICRA.* 2336-2341 (*2006*).

38 Meisel, D. V., Kuba, M., Byrne, R. A., Mather, J. The effect of predatory presence on the temporal organization of activity in Octopus vulgaris. *Journal of Experimental Marine Biology and Ecology.* **447**, 75-79 (2013).

39 Nesher, N., Levy, G., Grasso, F. W., Hochner, B. Self-recognition mechanism between skin and suckers prevents octopus arms from interfering with each other. *Current Biology.* **24** (11), 1271-1275 (2014).

40 Wells, M. J. *Octopus : Physiology and behaviour of an advanced invertebrate*. Chapman and Hall. Halsted Press. (1978).

41 Young, J. Z. *The anatomy of the nervous system of Octopus vulgaris*. Clarendon Press. (1971).
42 Zullo, L., Sumbre, G., Agnisola, C., Flash, T., Hochner, B. Nonsomatotopic organization of the higher motor centers in octopus. *Current Biology.* **19** (19), 1632-1636 (2009).
43 Albertin, C. B. et al. The octopus genome and the evolution of cephalopod neural and morphological novelties. *Nature.* **524** (7564), 220-224 (2015).
44 Albertin, C. B., Simakov, O. Cephalopod Biology: At the intersection between genomic and organismal novelties. *Annual Review if Animal Biosciences.* **8**, 71-90 (2020).
45 Baik, S. et al. A wet-tolerant adhesive patch inspired by protuberances in suction cups of octopi. *Nature.* **546** (7658), 396-400 (2017).
46 Pikul, J. H. et al. Stretchable surfaces with programmable 3D texture morphing for synthetic camouflaging skins. *Science.* **358** (6360), 210 (2017).
47 Wehner, M. et al. An integrated design and fabrication strategy for entirely soft, autonomous robots. *Nature.* **536** (7617), 451-455 (2016).
48 McMahan, W. et al. Field trials and testing of the OctArm continuum manipulator. *Proceedings 2006 IEEE International Conference on Robotics and Automation, 2006. ICRA*. 2336-2341 (2006).
49 Hochner, B., Brown, E. R., Langella, M., Shomrat, T., Fiorito, G. A learning and memory area in the octopus brain manifests a vertebrate-like long-term potentiation. *Journal of Neurophysiology.* **90** (5), 3547-3554 (2003).
50 Tapia-Vasquez, A. E. et al. Proteomic identification and physicochemical characterisation of paramyosin and collagen from octopus (Octopus vulgaris) and jumbo squid (Dosidicus gigas). *International Journal of Food Science & Technology.* **55** (10), 3246-3253 (2020).
51 Kim, B.-M. et al. The genome of common long-arm octopus Octopus minor. *GigaScience.* **7** (11) (2018).
52 Zarrella, I. et al. The survey and reference assisted assembly of the Octopus vulgaris genome. *Scientific data.* **6** (1), 13-13 (2019).
53 Forsythe, J. W., Hanlon, R. T. Effect of temperature on laboratory growth, reproduction and life span of Octopus bimaculoides. *Marine Biology.* **98** (3), 369-379 (1988).
54 Stoskopf, M. K., Oppenheim, B. S. Anatomic features of Octopus bimaculoides and Octopus digueti. *Journal of Zoo and Wildlife Medicine.* **27** (1), 1-18 (1996).
55 Ramos, J. E. et al. Body size, growth and life span: implications for the polewards range shift of Octopus tetricus in south-eastern Australia. *PLoS One.* **9** (8), e103480 (2014).
56 Hanlon, R. T., Forsythe, J. W. Advances in the laboratory culture of octopuses for biomedical research. *Lab Animal Science.* **35** (1), 33-40 (1985).
57 Moltschaniwskyj, N. A., Carter, C. G. Protein synthesis, degradation, and retention: mechanisms of indeterminate growth in cephalopods. *Physiological and Biochemical Zoology.* **83** (6), 997-1008 (2010).
58 Fiorito, G. et al. Guidelines for the care and welfare of Cephalopods in Research -A consensus based on an initiative by CephRes, FELASA and the Boyd Group. *Lab Animal.* **49** (2 Suppl), 1-90 (2015).
59 Valverde, J. C., Garcia, B. G. Suitable dissolved oxygen levels for common octopus (Octopus vulgaris cuvier, 1797) at different weights and temperatures: analysis of respiratory behaviour. *Aquaculture.* **244** (1-4), 303-314 (2005).

60 Cardeilhac, P. T., Whitaker, B. R. Copper Treatments: Uses and Precautions. *Veterinary Clinics of North America: Small Animal Practice.* **18** (2), 435-448 (1988).
61 Hodson, P. V., Borgman, U., Shear H. Toxicity of copper to aquatic biota. *Copper in the Environment (2)*. John Wiley. 307 - 372 (1979).
62 Poole, B. M. Techniques for the culture of ghost shrimp (palaemonetes pugio). *Environmental Toxicology and Chemistry.* **7** (12), 989-995 (1988).
63 Burggren, W. W. Respiration and circulation in land crabs: novel variations on the marine design. *American Zoologist.* **32** (3), 417-427 (1992).
64 Reitsma, J., Murphy, D. C., Archer, A. F., York, R. H. Nitrogen extraction potential of wild and cultured bivalves harvested from nearshore waters of Cape Cod, USA. *Marine Pollution Bulletin.* **116** (1), 175-181 (2017).
65 Messenger, J. B. Cephalopod chromatophores: neurobiology and natural history. *Biological Reviews.* **76** (4), 473-528. (2001).
66 Holst, M. M. Miller-Morgan, T. The Use of a species-specific health and welfare assessment tool for the giant pacific octopus, enteroctopus dofleini. *Journal of Applied Animal Welfare Science.* **24** (3), 272-291 (2021).
67 Rosas, C. et al. Energy balance of Octopus maya fed crab or an artificial diet. *Marine Biology.* **152** (2), 371-381 (2007).
68 Anderson, R. C., Wood, J. B., Byrne, R. A. Octopus Senescence: The Beginning of the end. *Journal of Applied Animal Welfare Science.* **5** (4), 275-283 (2002).
69 Butler-Struben, H. M., Brophy, S. M., Johnson, N. A., Crook, R. J. In vivo recording of neural and behavioral correlates of anesthesia induction, reversal, and euthanasia in cephalopod molluscs. *Frontiers in Physiology.* **9**, 109 (2018).
70 Jiang, X. et al. Octopus visual system: A functional MRI model for detecting neuronal electric currents without a blood-oxygen-level-dependent confound. *Magnetic Resonance in Medicine.* **72** (5), 1311-1319 (2014).
71 Hennig, J., Nauerth, A., Friedburg, H. RARE imaging: a fast imaging method for clinical MR. *Magnetic Resonance in Medicine.* **3** (6), 823-833 (1986).
72 Brant-Zawadzki, M., Gillan, G. D., Nitz, W. R. MP RAGE: a three-dimensional, T1-weighted, gradient-echo sequence--initial experience in the brain. *Radiology.* **182** (3), 769-775 (1992).
73 *emit-Xerra*, <http://emit-imaging.com/xerra/> (2021).
74 Bertapelle, C., Polese, G., Di Cosmo, A. Enriched environment increases PCNA and PARP1 Levels in Octopus vulgaris central nervous system: first evidence of adult neurogenesis in Lophotrochozoa. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution.* **328** (4), 347-359 (2017).
75 Maselli, V., Polese, G., Soudy, A.-S. A., Buglione, M., Cosmo, A. D. Cognitive stimulation induces differential gene expression in Octopus vulgaris: The key role of protocadherins. *Biology (Basel).* **9**, (2020).

Figure 1

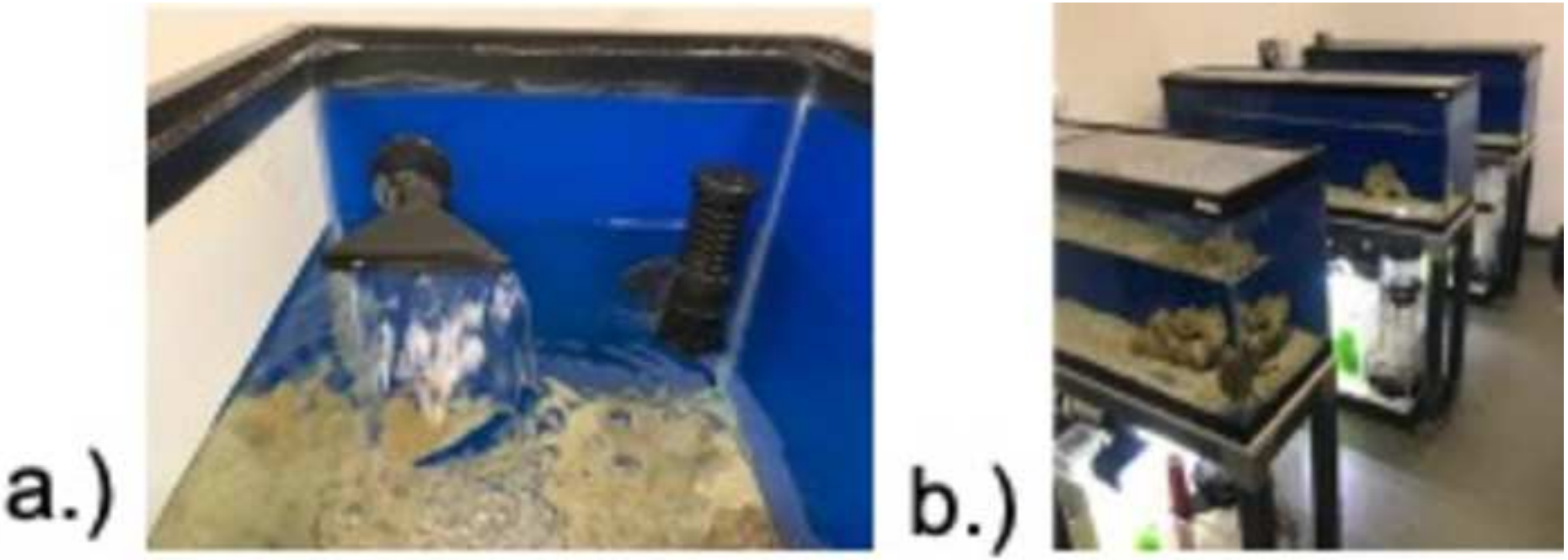
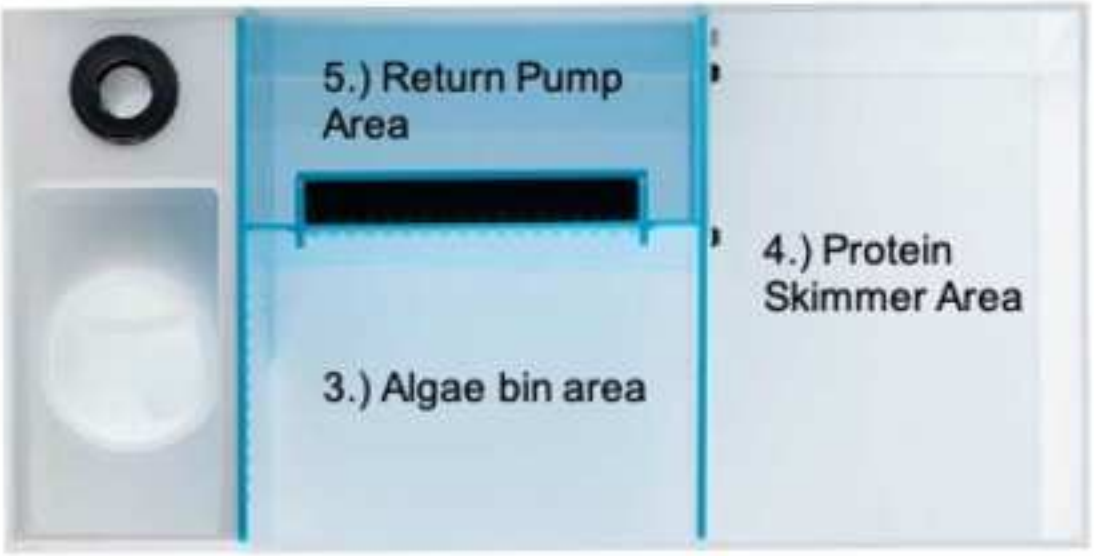


Figure 2

a.) Front View



b.) Top View

Figure 3

Click here to access/download;Figure;Figure_3.jpg

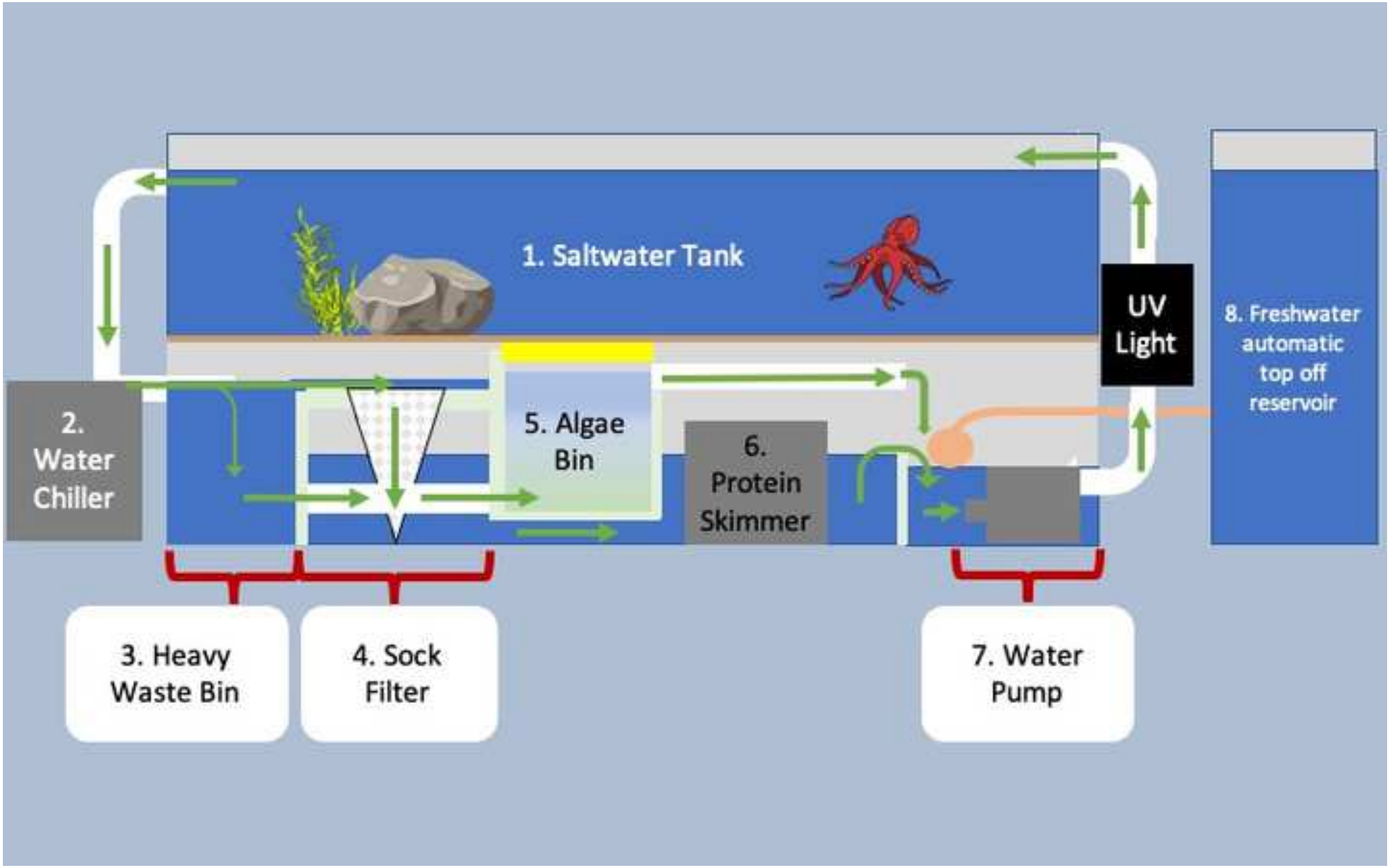


Figure 4

Click here to access/download;Figure;Figure_4.jpg

Figure 5

Click here to access/download;Figure;Figure_5.jpg

Figure 6

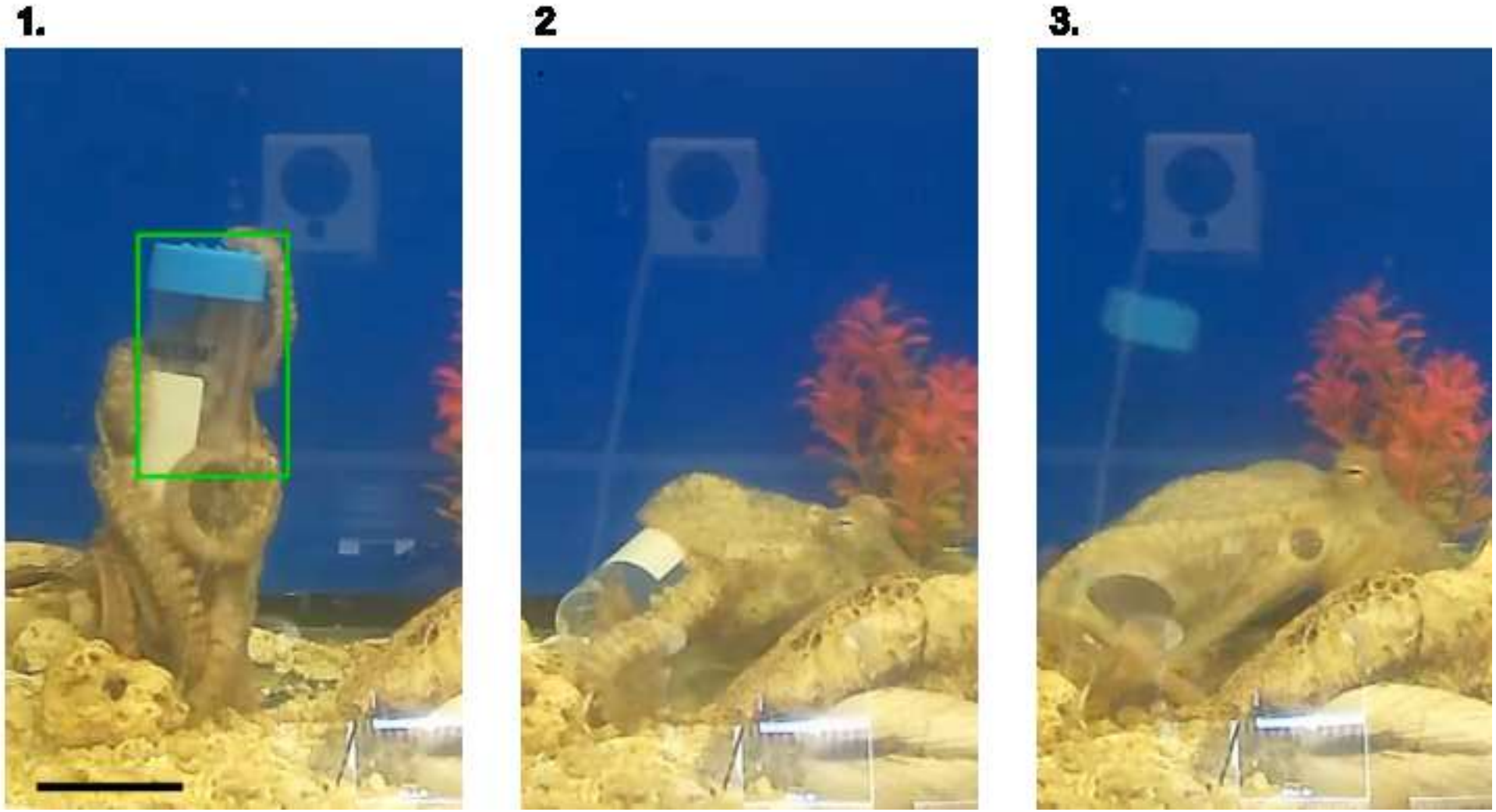


Figure 7

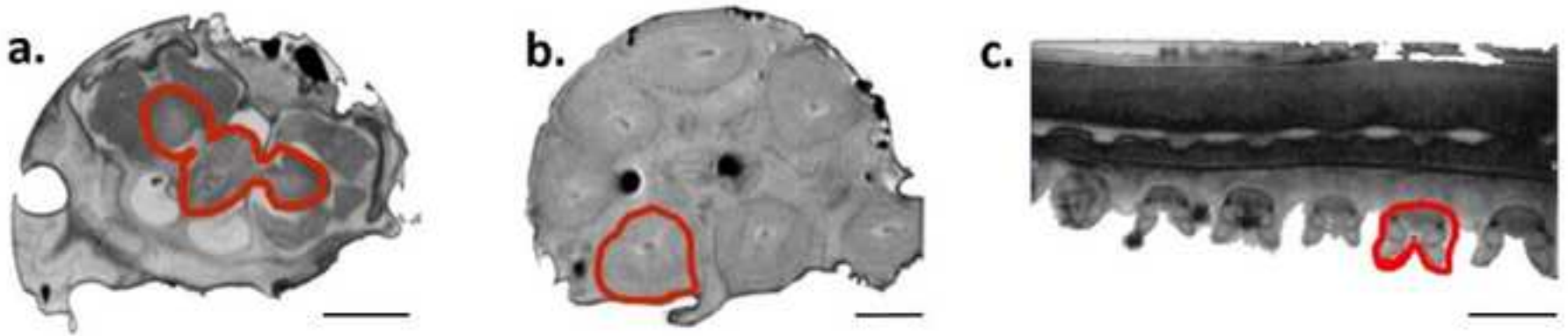
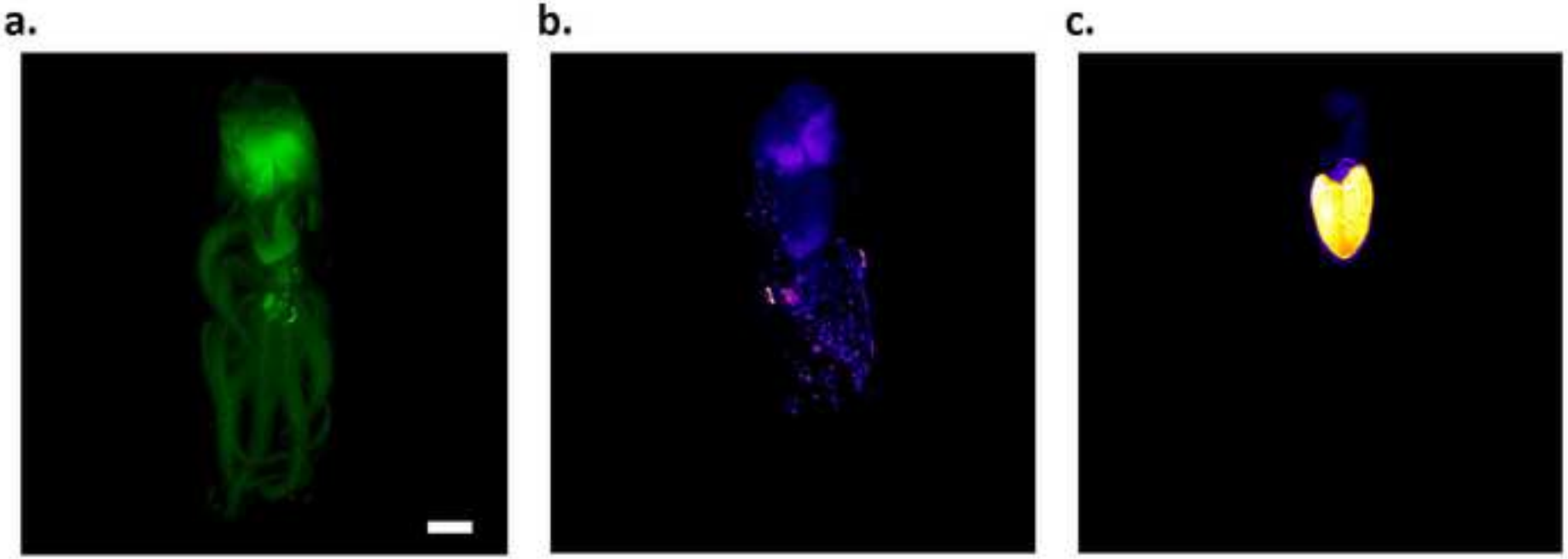


Figure 8

Click here to access/download;Figure;Figure_8.jpg



Click here to access/download

Table of Materials

Copy of Octopus_JoVE_Materials_08-26-2021.xls

Dear Editor and Reviewers,

We appreciate and extremely thankful for your thoughtful feedback. We have addressed all the revisions suggested by the reviewers. Please find them below point by point (in blue).

Reviewers' comments:

Reviewer #1:

Major Concerns: None

Minor Concerns:

1. Line 23, "octopus's" is not correct, change to " octopus' ". The same mistake appears in lines 72, 76, 84, and 671.

Thank you for noticing this issue, it was corrected.

2. Line 57-59, octopus is used here as genus, please change to Octopus (in italics).

Corrected.

3. Line 78, "cephalopod family" is incorrect, change to "class Cephalopoda". Or rewrite the sentence: "octopus and other cephalopods like cuttlefish and squid".

Thank you for the suggestion it has been added.

4. Line 82-83, suggested: "that have inspired the development of several innovations in the engineering field, for example:"

Corrected.

5. Line 94, add a comma after "octopus". Add "that" before "can be found".

Corrected.

6. Line 97-98, change to "giant Pacific octopus", "common octopus", and "California two-spot octopus".

Corrected.

7. Line 98-99, what do you mean with "starting out smaller than a quarter"? is a quarter a US coin? Please, use metric system units when describing the animal size, so that an international reader can understand.

Thank you, it has been changed to smaller than a few centimeter.

8. Line 103, change "if housing more than a gender" to "if housing both sexes together". "Gender" is not an appropriate term for animals. Also, suggested change: "they are housed individually in separate tanks".

This has been added, thank you for your suggestion.

Protocol:

9. The reviewer suggests providing the material list before the protocol, and then explaining in the protocol how are the materials used. It is important to know all the materials required before anything else.

Thank you this has been modified to reflect suggested changes.

10. Please enumerate the materials used accordingly with their appearance in the protocol. The first part mentioned in the protocol should be "Part #1", not "Part #71", and then the "Part #2" not "Part #72" (However, this suggestion should be approved by the Editor). Besides, provide an approximate cost of the tank including all parts required.

We have discussed this with the editor who asked for the parts to appear in alphabetical order on the material's sheet.

11. Lines 118 and 121, what do you mean by "flesh with sides of a table"? The reviewer does not understand.

Corrected.

12. Line 183, "2 in above" should be "2 inches above"?

Corrected.

13. Line 196, "pip" should be "pipe"?

Corrected.

14. Lines 211-213, the reviewer does not understand this instruction, is it correct? Please check.

A figure has been referenced to give the reader an example of what is being done.

15. Line 214, the "bulkhead" word repeats.

Corrected.

16. Line 244-245, please check the correctness of this sentence, "no additional freshwater is unnecessarily added". The reviewer suggests: "no additional freshwater is required".

The suggested change has been added, thank you.

17. Line 280-281, write the common names of species in lowercase letters (i.e., spaghetti algae, cherrystone clams)

This has been changed, thank you.

18. Line 312, "Add an over the side" … it seems that a word is missing.

The sentence has been modified to the following: “Attach cannister filter to the edge of tank”

19. The whole protocol needs grammar and orthographic corrections. Double-check the clarity of the instructions, and the correctness of the punctuation marks. Some paragraphs were difficult to understand.

We addressed this, thank you for your comment.

Results:

20. Report the measurements of the main physiochemical water parameters through time (pH, salinity, O2, temperature, ammonia, etc); how stable or variable the system was?

This was added within the discussion section: “The average recorded parameters for a tank over a week were pH 8.4 std 0.0, salinity 34.06 std 0.61 ppt, temperature 18.7 std 0.75 °C, ammonia 0.11 std 0.14 ppm, nitrite 0.25 std 0.14 ppm, and nitrate 1.43 std 2.44 ppm.”

21. Provide results about the general octopus health, presence or absence of parasites, bacteria, skin damage, or abnormal behaviors. Also, provide initial and final wet weights of all octopuses used in this study or provide growth rates.

Octopus condition was observed daily. We did not see parasites, bacteria, skin damage, or abnormal behavior. Octopuses arrived from the wild at ages that were unknown to us, and their stay in the lab was variable. Thus we do not have comprehensive data on growth rate.

22. Please provide an estimate for the daily or weekly costs to maintain one average-size octopus, including feeding, electric energy required for the whole system, prey maintenance, etc.

Charges associated with system maintenance varied considerably every week, depending on local vendors, shipping availabilities, and other factors. Therefore, we cannot provide definitive numbers.

23. All the reported results must be discussed using recent literature.

Thank you we added literature.

24. The reviewer recommends omitting the MRI and CFT protocols since their results were not used to test any hypothesis. These procedures seem quite disconnected from the main goal of establishing an adequate and stimulating aquatic environment for octopuses. If authors would like to report the results derived from these procedures in another hypothesis-driven study, please do not mention them here. Besides, these procedures were not mentioned in the discussion, so it makes no sense to report such procedures and their results. A well-written, detailed, reproducible, and effective protocol that accomplishes the main objective (the establishment of an enriched environment for octopuses) could be publishable without additional (unnecessary) procedures that fall out of the main goal.

We appreciate your advice. The editor suggested that we will keep this section as written.

25. Line 455-459, change "end of life cycle" to "the senescence". Also, change "Male octopuses beginning end of life cycle will decrease food consumption" to "Senescent males will decrease food consumption".

The suggested change has been added.

26. Line 596, write "O. bimaculoides" in italics.

Corrected.

27. Line 673, what is a "one-way house"?

This has been changed to “house with one entrance”

28. Line 684, change "edible animals" to "preys", and give some examples.

Additional improvements would be to introduce different types of algae to control nitrate levels as well as other prays, such as other non-poisonous mollusks and decapods, that the octopus may prefer as additional options.

29. Line 687-688, the authors mentioned that the protocol provided a stable and healthy environment. However, no results are demonstrating the stability of the system nor the health of the animals. It is very important to report and discuss those results.

Thank you we addressed this in comment 20.

Reviewer #2:

Manuscript Summary:

This manuscript describes how to build and maintain a set up for holding octopuses in a lab for research purposes, as well as how to care for the animals in captivity.

Minor Concerns:

1. 147: It may be useful to mention that although PVC glue takes 24 hours to fully cure, it does set firm in well under a minute, it would convey to people that you really mean as quickly as possible!

This has been added thank you.

2. 486: Respiration rate is missing here, above what per minute?

This has been changed to: “Prior to moving the octopus, record octopus’ skin pigmentation and baseline breathing rate. Remove octopus from tank by using a clean 4 L open mouth container by scooping it up with its surrounding water.

NOTE: During anesthesia, breathing rates does not necessarily indicate complete anesthesia.”

“If respiration remain above baseline and animal continues to respond to a light pinch, add an additional 0.25% EtOH to water.”