NEURON TO NETWORK - RESEARCH INTO OCTOPUS NEUROBIOLOGY AND LABORATORY SETUP

By

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ABSTRACT

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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, and prosthetics. To begin research with this species of cephalopods the set-up of complex facilities and intensive care routines for both the octopus and its ecosystem is predicated on the project's success. After the successful deployment of this marine ecosystem, research into the neurobiology of the octopus's limbs began with the study of provoked responses in extracellular ex vivo tissue through electrical stimulation. The preliminary results suggest that the methods employed within this study show extracellular electrophysiology data can be recorded from the tissue as well as evoke responses. Outcomes from this research assist to understand how to successfully record electrophysiological data from octopuses and effectively analyze the results to support next steps in the research. The data collected from this study will be employed for testing in vivo recording electrodes for the eventual goal of understanding how an octopus's nervous system coordinates movement while engaging in normal behavior. This will ultimately aid in replicating its mechanisms for locally controlled movement through robotics for the use in prosthetics.

ACKNOWLEDGMENTS

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INTRODUCTION

As a result of the innovation of new assistive technologies, treating neurological disorders, and brain machine interfaces, many other investigators have been able to examine the physiology of other species to improve the standard of living for individuals in the human population. Advancements in robotics, prosthetics, and computational modeling have been made possible by using octopuses as an animal model [1-3]. Examples of advancements made thanks to octopus research include a miniature soft octopus robot called 'octobot' that can function as surgical tools within the body [13], a material that functions like an octopus sucker to grip and hold objects [14], programmable camouflaging material made to mimic octopus skin to change with its surroundings [15], and underwater drone that uses a syphon for propulsion [16]. Octopuses have a complex integration of multiple types of sensory receptors, infinite degrees of freedom for movement, and unique reflex pathways making them optimal for developing novel technologies like the ones described above. There are many species of octopuses used in research that include the *Octopus Vulgaris*, *Octopus Sinens*is, *Octopus Variabilis*, and *Octopus Bimaculoides* where the *Octopus Vulgaris* and *Bimaculoides* are the most common to be used in biomedical research due to the depth of their documented anatomy and physiology [4-8]. Furthermore, our research focuses on the *Octopus Bimaculoides* due to its recently sequenced genome that will benefit other research questions within our lab [9].

The octopuses are a unique species with its nervous system containing approximately 45 million neurons in its central brain, 180 million neurons inside its optic lobes, and 350 million neurons distributed among its eight arms. Based on previous research, this anatomical dedication of

neurons to each arm plays an important role in allowing limited decentralized control of the arms to provide distinctive functionality to each limb making the octopus the best animal model for research into the control that goes into individual arm movements [8, 14].

Improving the understanding of how the octopus's neurobiology allows the control of their arms using this unique paradigm has been the chief objective of my research. To perform this study of their neurobiology a team, including myself, designed, tested, and deployed four aquariums with robust environmental parameter monitoring and controls. These ecosystems allowed our team to study the behavior and nervous system of the *Octopus Bimaculoides* (Bimac). Our main research focus was the mechanisms of control the octopus employs for each of their eight arms as well as how this local control integrates with their whole-body movements such as swimming and crawling. To begin studying the connection between neurological activity and movements it has been my focus to be able to record neurological data from octopus tissue, define what constitutes and action potential, and determine if there are underlying patterns. First answering these questions will assist in developing a method of studying this network of peripheral cellular interactions and correlating them with octopus movements.

Aim 1 of my research was the setup of complex facilities and intensive care for both the octopus and its ecosystem that is critical for the overall 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 is required to effectively keep the facility operating long term.

Aim 2 of my research was to determine the differences in neurological responses between peripheral stimulation and axial stimulation within an octopus arm. This research will be done using multi-electrode array to perform extracellular recording within a transverse slice of tissue removed from the octopus arm.

The results obtained from my research aims will inform future studies in our lab to eventually link specific neurological activity of octopuses with the exact action that follows. This research will assist in advancing responsive prosthetics as well as robotics that utilize brain machine interfaces (BMI) through enhanced algorithms for controlling external devices [10-12]. Even with the significant recent improvements in these devices, they still do not provide enough precise control of the prosthetic limb or any sensory feedback, but these obstacles could be overcome as a result of future research regarding octopus nervous system structure and limb movement. Improved motor planning is one significant field this project will work to develop and improve on.

This study will improve upon fields of neurological research, biomechanical engineering, and marine biology by providing a greater insight into octopus neurobiology and how this animal utilizes its dynamic movement mechanisms. The wider context of this research is that it will eventually assist in the development of prosthetics that can produce a gripping motion,

working to reproduce the numerous mechanisms an octopus' uses for this action. With 2.5 million people living with amputations in the United States and another 57.7 million worldwide, the need for improved technologies that can assist in regaining mobility is necessary [13].

CHAPTER 1: OCTOPUS CARE

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 [13]; identifying ion channels activated by light in algae has led to the development of cellular and temporal specific light-based-neuromodulation [14-17]; discovering proteins in glass catfish that navigate according to the Earth's magnetic field has led to the development of magnetic-based-neuromodulation [18-23]; understanding the siphon reflex in *Aplysia* has been instrumental to understanding the cellular basis of behavior [24-26].

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*. Indeed, many aspects of octopus' physiology and behavior have been studied over the past decades [2, 4, 27-36]. 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 [3, 5, 37-47].

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. 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[8, 48, 49]. 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[9, 50] 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[51]; a synthetic camouflaging, octopus skin that can transform a flat, 2D surface to a three-dimensional one with bumps and pits[52]. Miniature soft and autonomous robots (i.e., Octobots) that in the future could serve as surgical tools inside the body[53]; and an arm (i.e., OctoArm) attached to a tank-like robot[54] 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[5, 6, 55]. The recent sequencing of different octopus genomes makes this genus of particular interest and opens new frontiers in octopus research [5, 9, 56, 57].

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 [29]. 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[58, 59]. 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 [60-62]. Octopuses can be cannibalistic, especially if housing both sexes together within a tank; therefore, they need to be housed individually in separate tanks [63].

METHODS

Octopus Tank Equipment Set-Up

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** in **Appendix A**. Sizes are provided in inches.

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.

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.

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 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.

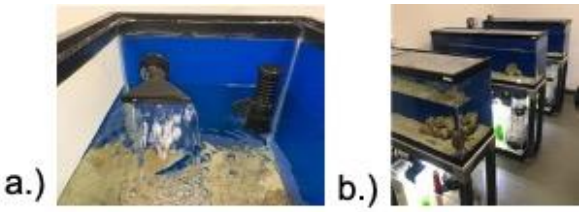

Figure 1: Octopus tank setup. Water inlet and outlet (a). Three octopus tanks each with an area of 1.22 m x 0.3 m (b).

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.

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.

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.

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.

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.

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.

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.

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

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.

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**.

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.

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.

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.

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.

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).

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.

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.

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.

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).

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**).

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.

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.

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.

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[58]. Turn on the protein skimmer.

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.

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[63].

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.

32. After the system is established, also check that the pH and oxygenation is at 8.0–8.4 and $4.5 \pm 0.95 \frac{mgO_2}{L}$ [64], 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[65, 66].

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.

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.

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).

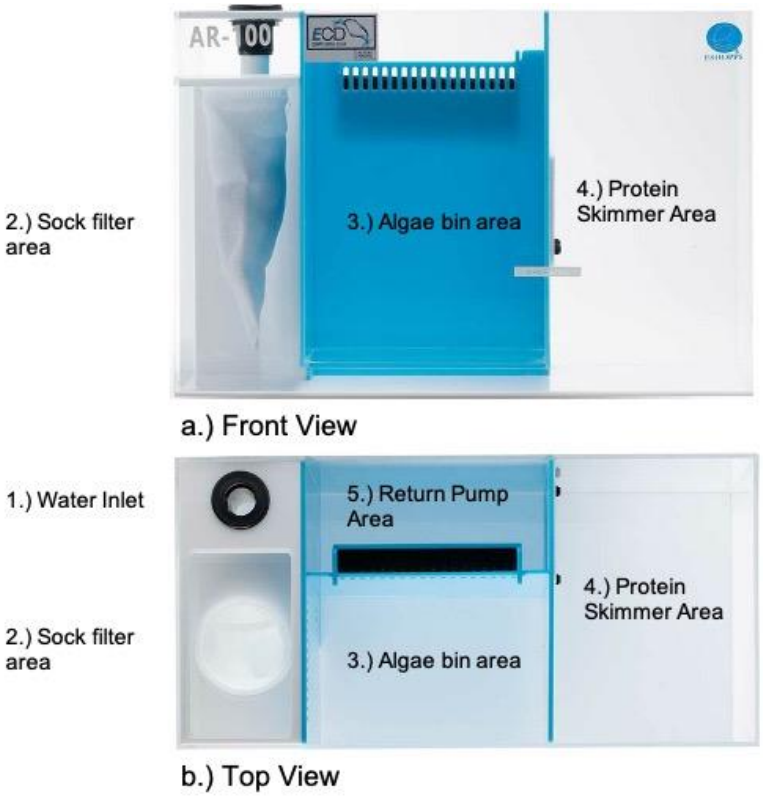

Figure 2: Sump system. Side view of the sump system (a). Top view of the sump system (b).

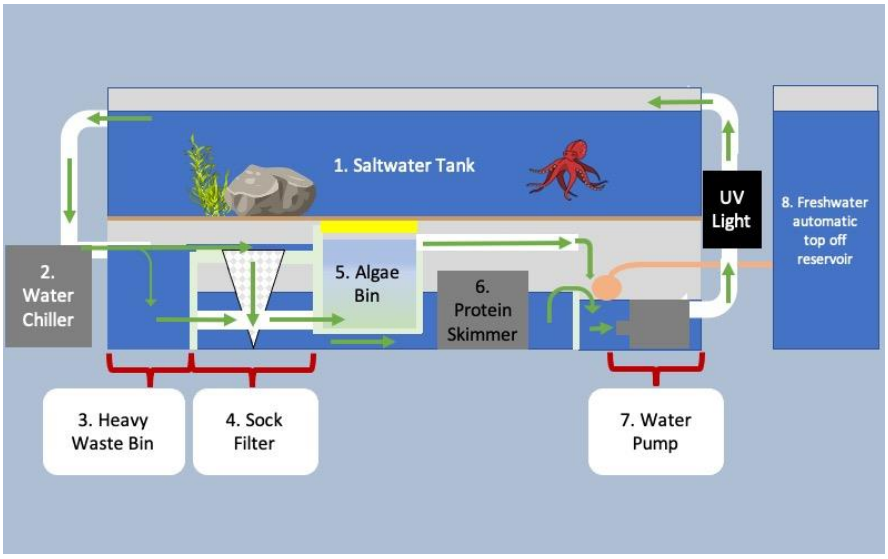

Figure 3: Aquarium with sump filtering system below the tank and environmental control units. Green arrows indicate direction of water flow through the system. Water flowing from section one to two for cooling and onto three to separate heavy biological matter from lighter matter. Heavy waste floats 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.

Storage tanks

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. 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.

Food tank setup

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.

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. 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.

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.

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.

6. Feed shrimp and fish with fish flakes, dead vegetation, or algae [67], as directed on food instructions.

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 [23, 68], respectively.

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.

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.

10. Store marine bivalve mollusks (clams and mussels) within the saltwater tanks for the octopuses to open themselves and provide another water filtering mechanism[69].

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.

Introduction of octopus to the tank

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.

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.

3. 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. 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.

5. 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.

6. For the first 2 weeks after arrival, monitor the octopus' daily consumption that should be around 4% to 8% of their weight [63, 70, 71]. 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.

Daily care

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.

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.

1. 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.

2. 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 [72]. Frozen shrimp can also be provided as a food source based on the octopus' weight.

3. 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.

Weekly sanitation

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.

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.

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.

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.

Care of unwell animals

1. Follow the guide reference [71] 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[73].

Octopus anesthesia

1. Perform octopus anesthesia as detailed in Butler-Struben et al [74].

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 [63].

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.

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.

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.

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

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. 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.

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.

Octopus euthanasia

1. Follow the international standards for octopus euthanasia as detailed in Fiorito et al., Moltschaniwskyj et al., and Butler-Struben et al [62, 63, 74].

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.

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.

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 [63]. 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 [75, 76].

Future 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.

CHAPTER 2: OCTOPUS ELECTROPHYSIOLOGY

INTRODUCTION

Octopuses possess a decentralized control of their arms to perform many tasks with little input from their central brain. These tasks include grabbing food or propelling themselves through the water, and while these actions can be centralized, the execution of these tasks comes from neuronal activity in each individual [77, 78]. The aim of our project was to determine the neurological patterns that make it possible for the octopus to perform these movements, such as grasping, using neurons located within their limbs. To accomplish this goal, we will eventually utilize *in vivo* electrophysiology within a non-anesthetized octopus to find patterns between specific movements and neurological activity and eventually replicate it within robotics.

The first step I was tasked with for my own research was to record *ex vivo* cortical slices of the octopus arm using electrophysiology to determine if current methods in the field could keep the tissue alive, as well as to look for any potential differences between peripheral tissue stimulation and axial cord stimulation within the octopus arm. While electrophysiological recordings have been done to gain insights into their anatomy and physiology through studies such as anesthetics and euthanasia techniques [79], photosensitive vesicles [1], peripheral skin receptors [80], oculomotor cortex [81], and neurotransmitters [82] most extracellular recordings have been done before on the octopus brain, and few have been done on their limbs.

To start I began working to distinguish the differences between activity in different parts of the arm to get a baseline for neurological events as well as to determine how to characterize a pattern of activity within a limb. The basic pattern we are first working to define is activity originating from the peripheral vs axial chord. While this is a relatively simple first objective it incorporates multiple preliminary steps for accurately recording and analyzing the data collected from the tissue. To record these signals and activity, a relatively novel tool for recording electrophysiology from tissue was employed called a multi-electrode array. This array enabled me to record from 1048 electrodes and stimulate from up to 32 at once.

METHODS

Animals

The *Octopus Bimaculoides* used within the study were all adults, 6 months and older based on size upon arrival in the lab. These animals were purchased from a commercial vendor and shipped overnight to the laboratory. Animals were cared as described in **Chapter 1**. In this study, tissue experiments are only done on animals twice before being euthanized following the guidelines outline by University IUCUC protocols.

Equipment

The specialized experiment used for procedure is the MAXONE multi electrode array from Maxwell Biosystems. The recordings were sampled at 20kHz through Maxone software and analyzed using MATLAB software. Equipment used is shown in **Figure 6**.

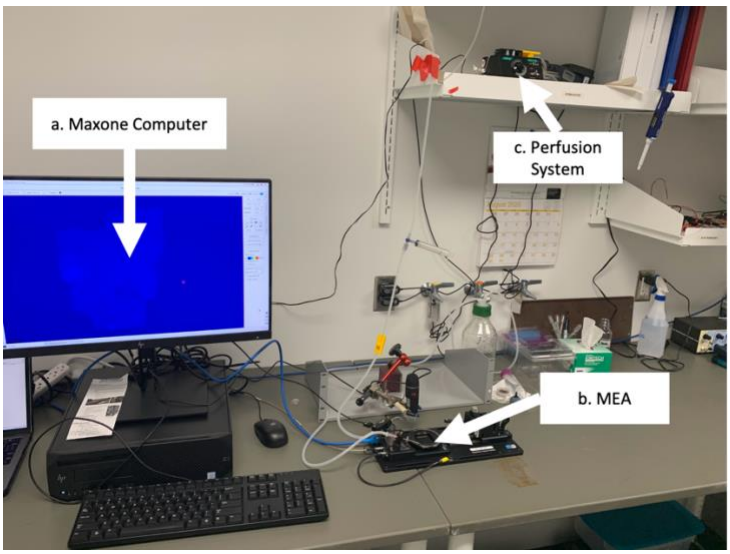

Figure 6: Maxwell Biosystems Maxone system setup. a.) Analysis computer with Maxone system interface. b.) Multielectrode array (MEA) where the tissue is placed onto array of 26000 electrodes, 1048 of them can be recorded from simultaneously, and Care2. c.) The perfusion system to move the extracellular octopus recording solution (EORS) solution overtop of the tissue while performing electrophysiology recordings.

Experimental Procedure

Tissue Preparation

First surgical equipment was autoclaved and extracellular octopus recording solution (EORS) was used to keep the collected octopus tissue alive. The EORS solution contained NaCl, 460mM; KCl, 10mM; MgCl2, 55mM; CaCl2 11mM; Hepes, 10mM; glucose, 10mM; pH 7.6. Next mix EORS solution in a 1:1 mixture of L15 [33]. This solution was modeled after the formula developed in Dr. Benny Hochner's laboratory at The Hebrew University of Jerusalem [33]. After EORS

preparation is complete it is stored at room temperature (25 C) and oxygenated with room air (4.5±0.95 (mg O_2)/L) [33, 64].

The next step is the preparation of the anesthesia and euthanasia tanks for the experiments. The anesthesia tanks required to begin with a 1% mixture of ethanol and the euthanasia tanks had 4% MgCl2, both oxygenated with room air [79]. Only octopuses that has undergone the following procedure before was euthanized due to guidelines given by university.

Using a 4L container submerged inside its aquarium the octopus is placed inside of container and moved to the surgical area. As a predicate for the procedure to begin the octopus was weighed and breaths per minute were recorded to obtain a baseline breathing rate. After the octopus was transferred to anesthesia container it remains there for 5 minutes after which breathing, skin color, and response to mantle pinch. If octopus is still responsive to being lightly pinched and its breathing rate remains above the baseline breathing rate the concentration of EtOH within container is increased by 0.25% [79]. This continues every 5 minutes until the octopus is unresponsive to stimuli and breathing drops below recommended levels to begin procedure.

The arms are each numbered from the octopus' point of view there being 4 arms on the left and right sides and the front arms each being numbered at 1 (L1,L2,L3,L4,R1,R2,R3,R4). Choosing which arm to remove is based on behavior and physiology. It has been noticed that the octopus utilizes arms L4/R4 for crawling and arms L1/R1 and L2/R2 for hunting. Due to the

octopus' reproductive system being on R3 the arm that was removed in each initial surgery was L3 [48].

Next L3 arm was clamped down near the distal point of the arm and a transverse cut was performed near the base of the arm severing it from the octopus, but not to cut any of the mantle. The arm is removed and placed into the oxygenated EORS solution. For the survival surgeries, after arm is removed 0.10 mL of lidocaine is administered into the area where the tissue was removed to treat pain [79]. The octopus was then moved back to their aquarium and placed on a strict monitoring period for the next 48 hours.

Each survival surgery was only performed once, if the octopus was in its second surgery it was euthanized after, and all body parts were utilized for experimentation. For non-survival surgeries the octopus is moved directly from the anesthesia tank into a 4% MgCl solutions and left there for 5 minutes after breathing has ceased [79]. After which the body is sealed and placed inside a -80 °C freezer.

Electrophysiology setup

Maxwell MAXONE single well multi electrode array (MEA) was used for recording neurological activity during these experiments. 1 mm cortical slice of octopus tissue was placed on the MEA with axial cord positioned as close to the center of the array (**Figure 7**). Tissue was clamped down to array and EORS solutions was perfused across slice.

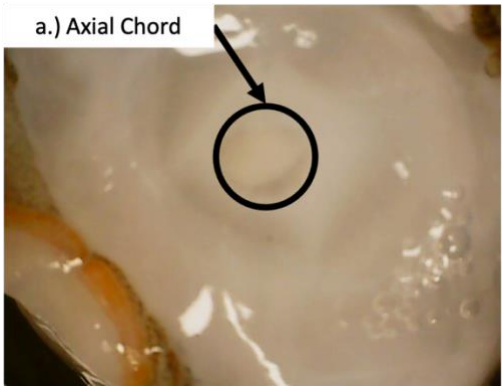

Figure 7: Octopus transverse slice of tissue. a.) Axial chord that runs down the center of the arm containing and eighth of the estimated 350 million neurons distributed among each of the eight arms. The tissue outside the circle is the peripheral tissue.

The first recording done on tissue was an activity scan. Activity Scan selects the most active electrodes across array where it believes are active neurons. After the first scan is complete a follow up network scan is then performed. Network scans allows to record activity from a large number of cells simultaneously. Based on the previously recorded Activity Scan a subsets of electrodes can be selected and recorded predicting possible connections between neurons based on firing rates and locations. These two scans are also done to find active electrodes and determine the likelihood that the tissue is alive and active.

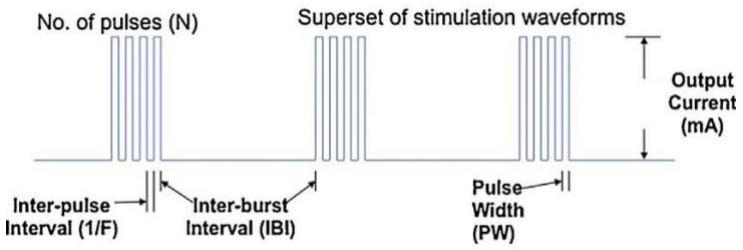

Figure 8: Diagram of key parameters of stimulation waveform. Diagram of how inter-pulse intervals, pulses, inter-burst intervals, bursts, and output current (amplitude) function as parameters for stimulations.

After initial scans are complete the next part is to begin performing stimulation recordings. This is done through a program that automatically performs a set of stimulation and records responses from 1048 electrodes. The stimulation parameters use both 9 electrodes or 32 electrodes creating a compact square for stimulating a wide area of tissue aiming to incite an action potential. Other recording parameters that were set for the stimulation where there were 100 pulses for each set of stimulations at a single voltage, 50 ms inter-pulse intervals (IPI), and 1000 ms inter burst intervals (IBI). The amplitude that the electrodes were stimulated at was increased in steps of 25 mV from 25 mV to 600 mV to determine the best stimulation range to use. A diagram for displaying the parameters is shown in **Figure 8**. Prior to adding tissue a recording was first done on the MEA with perfused EORS as the control.

RESULTS

We collected data from 11 of animals. Three to four slices were collected from each arm. Each stimulations paradigm was repeated 5-6 times. Altogether I collected 154 electrophysiology recordings of octopus tissue.

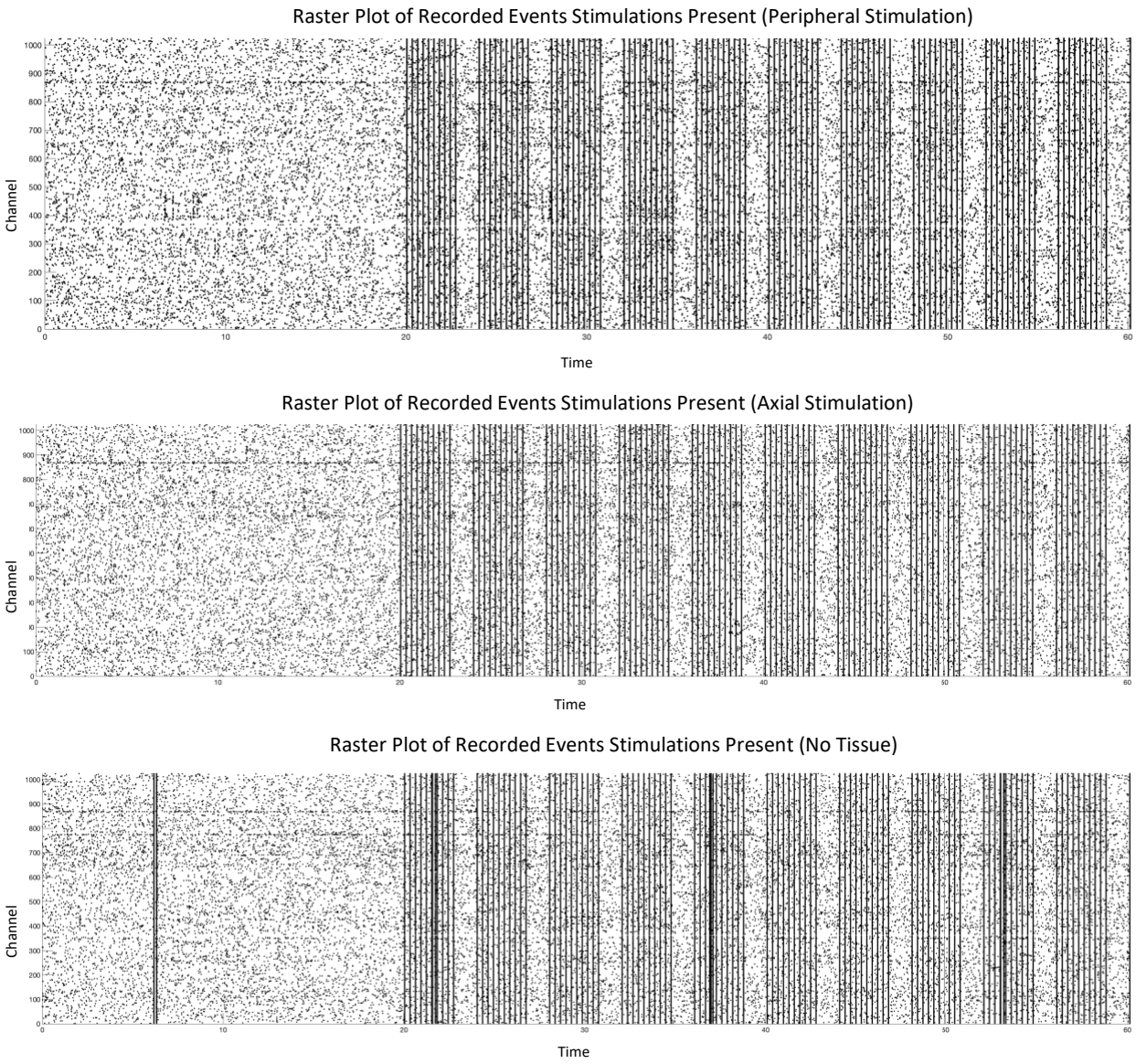

Figure 9: Temporal distribution of events recorded on the Maxone System. X-axis is time from 0 to 60 s and y-axis are each of the 1048 channels that recorded activity. The figures are representative conditions of the experiments.

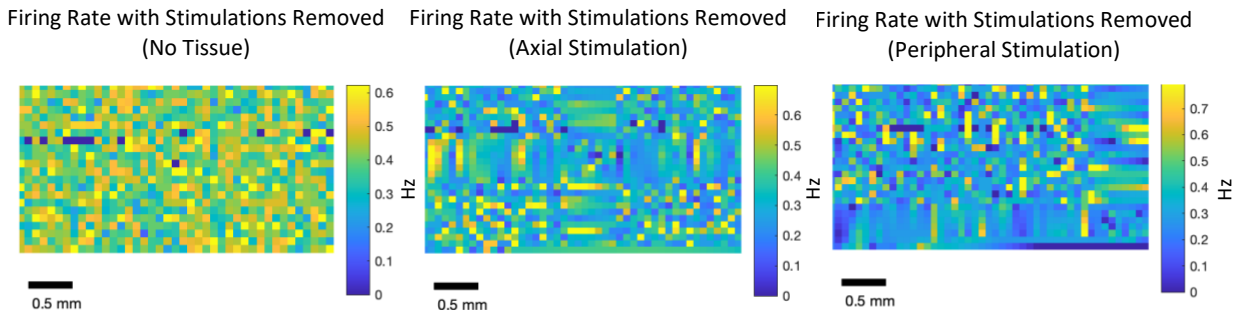

Figure 10: Spatial distribution of the firing rates. The three figures show the spatial distribution of the firing rates of each of the 1048 electrodes across the electrode array. These are shown the average results of the recordings with the stimulations removed post recording through data analysis software. The figures are representative conditions of the experiments.

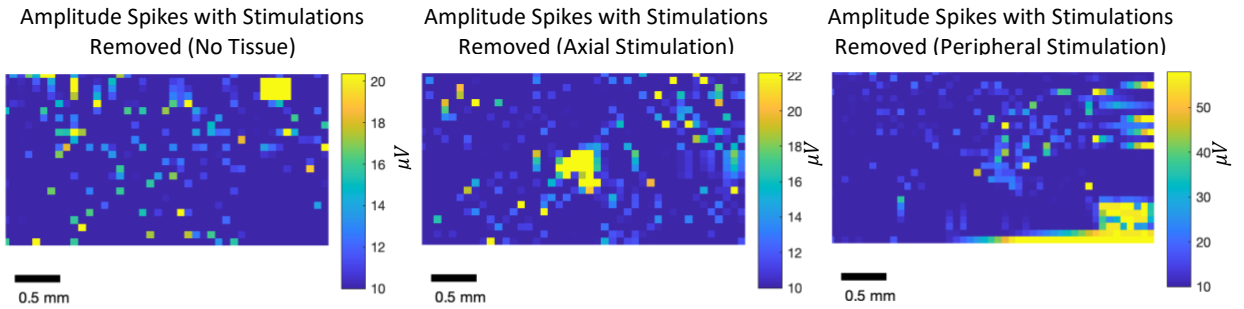

Figure 11: Spatial representation of the average amplitude. The three figures show a spatial representation of the average amplitude for each of the 1048 electrodes that were recorded from. Stimulations were removed post recording through data analysis software. The figures are representative conditions of the experiments.

DISCUSSION

The three metrics that we wanted to quantify during this project was to determine number of action potentials being recorded on each electrode, the temporal distribution the action

potentials (**Figure 9**), and where the there was an action potentials event (**Figure 10**). From group analysis of this information it would be determined if there is a specific pattern that is unique to processing information coming from the brain (axial cord stimulation) and from the periphery (peripheral stimulation). **Figures 8 - 11** are individual recordings that are representative of neural responses.

After generating the original recording, the signal was found to be saturated due to the stimulation and to resolve this issue the stimulations were removed through removing events that occurred simultaneously in groups of 500 or more and results of this is shown in **figure 10 and 11**. On further analysis of the spike amplitudes with the stimulations artificially removed, in **Figure 11**, shows a difference in mean spike amplitudes. The no tissue is 16.38 +/- 12.94 mV, axial stimulation 24.15 +/-66.80 mV, and peripheral stimulation 35.06 +/- 81.79 mV.

The results from the experiments allowed us to see the resulting activity from stimulations in different aspects through amplitudes, firing rates, and distributions of events. Thus far we only analyzed the individual traces of the 154 electrophysiological recordings. This is the first step towards completing these aims. The next step will be to perform a group analysis and determine if we can identify specific neural responses patterns. We anticipate that machine learning algorithms can be used to identify temporal and spatial characteristics associated with sensory motor circuits.

ETHICS STATEMENT

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

APPENDIX

Table 1: Animal Care Setup Material List.

Part Number	Name of Material/ Equipment
1	1-3/4 in. Drill Bit
2	1" flow sensors
3	1" Slip Bulkhead Strainer
4	10 gallon tank
5	4 inch X 12 inch 200 Micron Nylon Monofiliment Mesh Filter Sock w/ Plastic Ring
6	40 gallon aquarium
7	60g poly tanks - rectangle
8	Active Aqua 1/10th HP Hydroponic or Aquarium Chiller 2018 Model
9	ALAZCO 2 Soft-Grip Handle Heavy-Duty Tile Grout Brush
10	Ammonia Testing Kit
11	Apex system WiFi
12	API Aquarium Test Kit
13	API Copper Test Kit
14	Aqua Ultraviolet Classic UV 25 Watt Series Units
15	AquaClear 50 Foam Filter Inserts, 3 pack
16	Aqueon QuietFlow LED PRO Aquarium Power Filter 30
17	Auto Top Off Kit (ATK) (Each includes 1 FMM module, 2 optical sensors and 1 float)
18	Automatic top off from RODI (LLC)
19	Banded Trochus Snail
20	Chaetomorpha Algae, Aquacultured
21	Clams - Live, Hard Shell, Cherrystone, Wild, USA Dozen
22	Classic Sea Salt Mix - Tropic Marin
23	Clear Masterkleer Soft PVC Plastic Tubing, for Air and Water, 3/4" ID, 1" OD
24	CONTINUUM AQUABLADE-P ACRYLIC SAFE ALGAE SCRAPER W/ PLASTIC BLADE - 15 INCH
25	Copper Testing Kit
26	Curve Refugium CREE LED Aquarium Light
27	Eheim 1262 return pumps
28	Eshopps R-100 Refugium Sump GEN 3
29	Ethyl Alcohol, 200 Proof
30	Extech DO600 ExStik II Dissolved Oxygen Meter
31	Fiddler Crabs; live; dozen
32	Filter Cartrages
33	Florida Crushed Coral Dry Sand - CaribSea

Table 1 (cont'd)

34	FMM module
35	Fritz-Zyme TurboStart 900 - Fritz
36	Hand Operated Drum Pump, Siphon, Basic Pump with Spout, For Container Type Bucket, Pail
37	High pH Testing Kit
38	Imagitarium Fine Mesh Net for Shrimp
39	Leak Detection Kit (LDK) - Includes FMM module plus 2 ALD sensors
40	LEE`S ALGAE SCRUBBER PAD JUMBO - GLASS
41	Live rocks
42	Long Bottle Cleaning Brush 17" Extra Long
43	Magnesium chloride
44	Magnetic Probe Rack
45	Marine Ghost Shrimp
46	Marineland C-Series Canister Carbon Bags Filter Media, 2 count
47	Nitra-Zorb Bag
48	Nitrate Testing Kit
49	Nitrite Testing Kit
50	Pawfly 2 Inch Air Stones Cylinder 6 PCS Bubble Diffuser Airstones for Aquarium Fish Tank Pump Blue
51	Penn Plax Airline Tubing for Aquariums –Clear and Flexible Resists Kinking, 8 Feet Standard
52	Plumbing with unions/valves plus 3/4" flex hose
53	PM1 module
54	Protein skimmer
55	PVC Apex Mounting board, grommets, wire mounts
56	PVC Regular Cement and 4-Ounce NSF Purple Primer
57	RODI unit
58	Salinity Probes
59	Seachem Pristine Aquarium Treatment
60	Seachem Stability Fish Tank Stabilizer
61	Set of lexan tops
62	Set of Various extended length aquabus cables
63	SLSON Aquarium Algae Scraper Double Sided Sponge Brush Cleaner Long Handle Fish Tank Scrubber for Glass Aquariums

Table 1 (cont'd)

64	Standard-Wall PVC Pipe Fitting for Water, 45 Degree Elbow Adapter, 3/4 Socket Female x 3/4 Socket Male
65	Standard-Wall PVC Pipe Fitting for Water, 90 Degree Elbow Adapter, 1 Socket Female x 1 Socket Male
66	Standard-Wall PVC Pipe Fitting for Water, Adapter, 1 Socket-Connect Female x 1 Barbed Male
67	Standard-Wall PVC Pipe Fitting for Water, Straight Reducer, 2 Socket Female x 3/4 Socket Female
68	Standard-Wall PVC Pipe Fitting for Water, Tee Connector, White, 1 Size Socket-Connect Female
69	Standard-Wall Unthreaded Rigid PVC Pipe for Water, 1 Pipe Size, 10 Feet Long
70	Standard-Wall Unthreaded Rigid PVC Pipe for Water, 3/4 Pipe Size, 5 Feet Long
71	Structural FRP Fiberglass Sheet, 48" Wide x 96" Long, 1/4" Thick
72	Structural FRP Fiberglass Square Tube, 10 Feet Long, 2" Wide x 2" High Outside, 1/8" Wall Thickness
73	Tank Sediment
74	Temperature probe
75	Tetra TetraMarine Large Saltwater Flakes for all Marine Fish
76	Tetra Whisper Aquarium Air Pump for 10 gallon Aquariums
77	Thick-Wall Through-Wall Pipe Fitting, for Water, PVC Connector, 1 Socket-Connect Female
78	Vectra s2 pump
79	Water Pump
80	Wyze Cam v2 1080p HD Indoor WiFi Smart Home Camera with Night Vision

Table 2: Animal post surgery documentation sheet.

Date:	Time:	Weight:	Arm(s) Amputated:	Notes:
Octopus ID:		Sex:	Arm length:	
IUF #:		Anesthesia Used/Quantity:		

Time After Surgery:	Breating Rate:	Pain Sensitivity:	Skin Color:	Wound Swelling:	Notes
10 Minutes					
20 Minutes					
30 Minutes					
1 Hour					
6 Hours					
1 Day					

Documenter: Initials:

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