

An implantable two axis micromanipulator made with a 3D printer for recording neural activity in free-swimming fish



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HIGHLIGHTS

- A two axis micromanipulator was manufactured with a 3D printer.
- The micromanipulator allowed the implantation of electrodes into fish.
- It increased the number of fibers accessible for implantation and recording time.
- It provided a low cost option for implanting electrodes into aquatic animals.

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ABSTRACT

Background: Chronically implanted electrodes allow monitoring neural activity from free moving animals. While a wide variety of implanted headstages, microdrives and electrodes exist for terrestrial animals, few have been developed for use with aquatic animals.

New method: A two axis micromanipulator was fabricated with a Formlabs 3D printer for implanting electrodes into free-swimming oyster toadfish (*Opsanus tau*). The five piece manipulator consisted of a base, body, electrode holder, manual screw drive and locking nut. The manipulator measured approximately 25 × 20 × 30 mm (l × w × h) and weighed 5.28 g after hand assembly.

Results: Microwire electrodes were inserted successfully with the manipulator to record high fidelity signals from the anterior lateral line nerve of the toadfish.

Comparison with existing method(s): The micromanipulator allowed the chronically implanted electrodes to be repositioned numerous times to record from multiple sites and extended successful recording time in the toadfish by several days.

Conclusions: Three dimensional printing allowed an inexpensive (<\$US 5 material), two axis micromanipulator to be printed relatively rapidly (<2 h) to successfully record from multiple sites in the anterior lateral line nerve of free-swimming toadfish.

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1. Introduction

In the last twenty years, chronic neural recording in free-moving terrestrial animals has been made possible due to advances in electronics and hardware necessary to miniaturize electrophysiology recordings devices to interface with the nervous system. Headstages, which affix to the skull, allow electrode positioning and stability while incorporating amplifiers and filters. Tethers and/or wireless telemetry devices allow neural recording with minimal

impact to the animal's behavior. Many of the early experiments were conducted on primates (Nicolelis et al., 2003), however hardware miniaturization led to deployments on smaller animals such as songbirds (Fee and Leonardo, 2001; Otchy and Olveczky, 2012), cockroaches (Guo et al., 2014) and bees (Duer et al., 2015). Aquatic animals represent greater challenges as the recording apparatus needs to be waterproofed for electrical continuity and animal survival. Additionally, the increased drag in fluid environments necessitates that these devices be small and streamlined. The opacity of the marine environment to radio signals or infrared light limits transmission of standard telemetry devices and the use of tethers can introduce further complications through entanglements.

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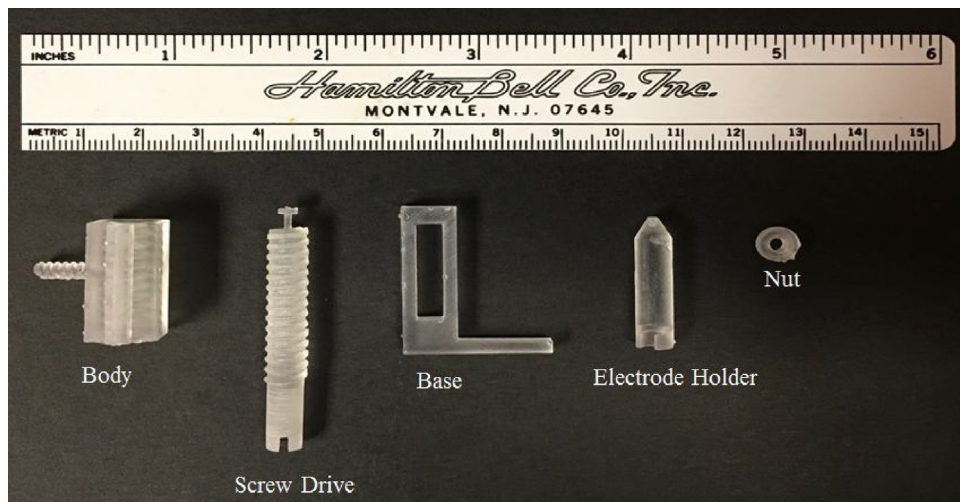


Fig. 1. The five unassembled 3D printed components of the micromanipulator.

Chronic neuronal recording often entails inserting electrodes into the cranial nerves or the brain. In the absence of a positioning device, the electrodes need to be firmly secured and often cannot be repositioned after implantation. Alternatively, electrodes can be repositioned after implantation if placed in miniaturized micromanipulators or microdrives, resulting in both a greater number of neurons and longer duration implants due to access to multiple neurons. However, recent advances in three-dimensional printing has seen the advent of compact devices that can be used in small species at a relatively low cost. For example, a 3D printed implant and assembly tool package (~3 g) with eight independently positional microdrives for neural recording and optical stimulation in freely moving mice recently has been developed (Freedman et al., 2016).

Headstages and microdrives for fishes have lagged behind development of recording technologies for terrestrial recording. One of the first attempts to record neural activity from free-swimming fish was reported using fixed electrodes in the goldfish telencephalon with multiunit recording reported up to five days (Canfield and Mizumori, 2004). A fixed electrode preparation recorded hindbrain activity in goldfish during C-starts, but recording was limited to approximately 6 h following implant (Weiss et al., 2006). Cerebellar neuron activity was also recorded in swimming goldfish, however the weight of the electrode and preamplifier impacted fish swimming and movement (Matsumoto et al., 2007). Recently, a wireless implant combined with a data logger that can store up to 2.5 h of data has been used to record from the optic tectum of goldfish and correlated neural activity with light stimulus (Vinepinsky et al., 2017).

One of the first reported chronically implanted devices to record neural activity from free-swimming fish was pioneered in the toadfish (Tricas and Highstein, 1990; Tricas and Highstein, 1991). The oyster toadfish is a benthic ambush predator that is characterized by a large flat skull. The cranium thins above the fore and mid-brain allowing access to several cranial nerves and the sensory end organs of the inner ear (semicircular canals and otoliths) which comprise both the vestibular and auditory systems in fish. The semicircular canals function as angular accelerometers while the otoliths act as both linear accelerometers and auditory end organs. The broad skull also provides sufficient area to anchor devices and the toadfish is exceptionally hardy as evidenced by several toadfish with implanted electrodes surviving space shuttle missions (Boyle et al., 2001). A uniaxial micromanipulator was developed to insert a single electrode into the anterior lateral line nerve (Tricas and Highstein, 1990; Tricas and Highstein, 1991). Later experiments

used a fixed, multichannel sieve microelectrode to record from the regenerating VIIIth nerve (Mensing et al., 2000). Multichannel microwire electrodes were implanted and coupled with an external inductive telemetry device or a tether to successfully record from the anterior lateral line and utricular nerve of the fish (Maruska and Mensinger, 2015; Mensinger, 2016; Palmer et al., 2005; Radford and Mensinger, 2014).

However, the microwire electrode needs to be fixed into place and cannot be repositioned after implantation. While units could be recorded for 48–72 h, the signals eventually fade and limited afferents can be assessed in each implant. The ability to reposition the electrode would provide longer windows for chronic recordings and the ability to regain lost units or access new ones. Additionally, the extended recording time could be used to monitor seasonal changes, short term effects of hormones, and exposure/recovery to environmental toxins.

To address this shortcoming, a 3D printed two axis micromanipulator was developed and its fabrication and ability to position microwire electrodes for recording high fidelity neural activity is described.

2. Materials and methods

2.1. Animal husbandry

Adult toadfish ($n=4$; 25 ± 2.7 SE cm standard length) were obtained from the Marine Biological Laboratory Woods Hole, MA. The fish were maintained in large flow through seawater tanks and maintained at local ambient seawater temperatures ($19\text{--}21^\circ\text{C}$). All experimental procedures conformed to institutional animal care protocols.

2.2. Micromanipulator

The 3D printed micromanipulator was designed using AutoCAD® 2016 software (Version M.48.M.617) (Autodesk®, San Rafael, CA, USA). The AutoCAD® design file was exported from *.dwg to *.stl format for printing. The micromanipulator was fabricated with a high resolution desktop Formlabs Form 2 3D printer (Somerville, MA, USA) with a resolution and build volume of $25\text{ }\mu\text{m}$ and $145 \times 145 \times 175\text{ mm}$, respectively. Formlabs clear photopolymer resin (Somerville, MA, USA) with a tensile strength of 65 MPa, elongation at failure 6.2% and flexure modulus 2.2 GPa was used for fabrication. Once fabrication was complete, the components of the micromanipulator were soaked in 95% ethanol

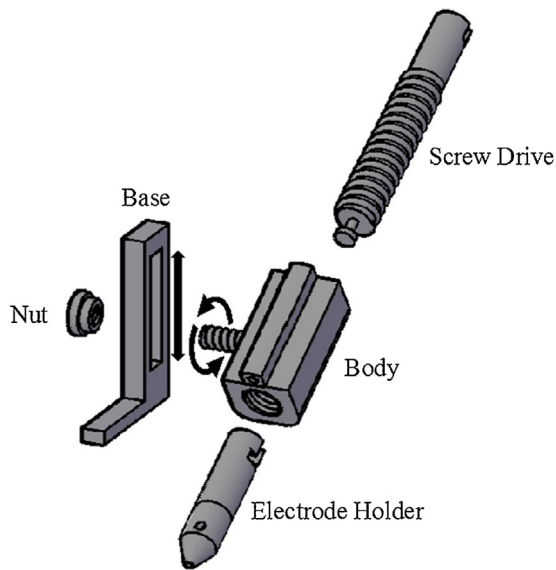


Fig. 2. An exploded view drawing of the micromanipulator.

for 20 mins to clean off any residual resin, rinsed in distilled water and air dried. The micromanipulator was then assembled and used with no post-cure treatments.

Five separate parts were printed and then hand assembled into each micromanipulator (Figs. 1 and 2). The one piece L shaped base consisted of a foot ($25 \times 5 \times 3$ mm) for attachment to the skull and 10×30 mm upright to support the body of the micromanipulator. The body of the micromanipulator measured $23 \times 21 \times 13$ mm with a threaded hollow core of 7 mm in diameter. A 10 mm long threaded rod (4 mm OD) projected from the body for insertion into the upright of the micromanipulator base. An electrode wire guide tube (4 mm OD; 2 mm ID) was integrated into the body during the printing and traversed the length of the body. The body was secured to the base upright with a round lock nut (8 mm OD; 4 mm ID). By adjusting the tightness of the nut, the body could be manually raised in the vertical plane as well as tilted 90° from vertical. The electrode holder was a 25 mm length rod (7 mm OD) which was tapered at the anterior end to a rounded point and with a partially hollowed groove at its posterior end. The holder contained a 20 mm long, 2 mm diameter central tube for the electrode wires to be threaded through. The central electrode tube was angled towards the outside at the 20 mm mark, to allow the electrode wires to exit the manipulator. The screw drive consisted of a solid rod, threaded on its outside, approximately 48 mm in length and 7 mm OD. The posterior end of the screw drive contained a groove for insertion of a flat head screwdriver to turn the drive during electrode insertion. A small 2 mm diameter stem terminated into a flat, circular disk (3 mm diameter) on the anterior end of the screw drive. The disk was part of a “lock and key” link between the drive and the electrode holder, and by inserting the disk into the back end of the electrode holder, the electrodes could be advance by manually turning the drive with a flat head screw driver or by hand, and during the advance, the disk would rotate in the groove without rotating the electrode holder. The assembled micromanipulator measured approximately $25 \times 15 \times 30$ mm and weighed 5.28 g (Fig. 3).

2.3. Microwire electrode

Microwire electrodes consisting of twin insulated $20 \mu\text{m}$ diameter 10% platinum/iridium wire (Sigmund Cohn) were custom fabricated for each implant. Each microwire was fixed to hard silver-plated copper multistranded wire ($25 \mu\text{m}$ diameter, New

England Wire) with conductive silver paint and then the multi-stranded wire was soldered to silver wire ($320 \mu\text{m}$). The anterior portions of the microwires were threaded through a 1 cm length of polyimide tubing ($180 \mu\text{m}$ OD) to maintain the recording sites in proximity. The silver wire was threaded through the micromanipulator electrode holder until the polyimide tubing was in position at the cone shaped end of the holder. A small drop of cyanoacrylate gel was used to glue the polyimide tubing to the holder. Miniature gold plated pins or alligator clips were soldered to the terminus of each of the wires that were protruding from the posterior end of the electrode holder. Any exposed wire/connectors were encased in Bondic UV activated clear glue and cured with ultraviolet light. The impedance of each electrode channel was determined with an impedance-test unit (FHC) and only electrodes with impedances between 0.5 and $1.5 \text{ M}\Omega$ were used.

2.4. Implant

Fish were anaesthetized by immersion in 0.005% tricaine (3-aminobenzoic acid ethyl ester) in seawater and paralyzed with an intramuscular injection of 0.01% pancuronium bromide ($600 \mu\text{g kg}^{-1}$). The fish was then placed in a custom designed stereotactic aquarium. An incision was made through the dorsal musculature overlying the sagittal crest, and the muscle bilaterally retracted. A small craniotomy was performed to the right of the sagittal crest and posterior to the transverse crest to expose the anterior ramus of the anterior lateral line nerve. The exposed portion of the sagittal crest was removed and the surface of the skull to the left of the midline was cleaned of tissue and fluid. The base of the manipulator was attached to the skull's surface with cyanoacrylate gel.

The height of the manipulator arm was adjusted vertically and the drive was positioned at the best angle to access the anterior lateral line nerve. The electrodes were inserted manually into the nerve by turning the screw drive. Once high fidelity neural recordings were confirmed, the craniotomy was sealed with cyanoacrylate glue (gel) that spanned the surface of the cranium to the outside body of the micromanipulator. The muscle, fascia, and epidermis were returned to their original position, covering the base and lower half of the manipulator so only the posterior end of the drive protruded through the skin. The muscle, fascia, and epidermis were each sutured separately and pulled tight around the manipulator to form a water tight seal. The final epidermal layer was also sealed to the outside body of the micromanipulator body with cyanoacrylate glue (Fig. 4).

Action potentials were differentially amplified (Dagan, USA) and monitored on a portable computer using Spike2 for windows software (Cambridge Electronic Design Ltd, UK). The fish was then transferred to the experimental tank and allowed to recover for 90 min. Following the recovery period, the three electrode wires were attached with a water proof connector to a 2.5 m long, flexible tether that terminated into the differential amplifier.

2.5. Experimental set-up

The experimental tank consisted of a plexiglass aquarium 1.0×0.67 m with water depth maintained at 10 cm which completely immersed the fish and protruding micromanipulator.

Spontaneous and mechanically evoked neural activity was recorded using ADInstruments powerlab. A small brush was run over the surface of the fish to pinpoint the location of the innervated lateral line neuromasts. Once a unit was confirmed to be responsive to mechanical stimulation, the electrodes were retracted from the nerve by turning the screw drive, and then the micromanipulator was reinserted to obtain additional units. Waveform analysis was performed on the data, using Spike2 software (Cambridge Elec-

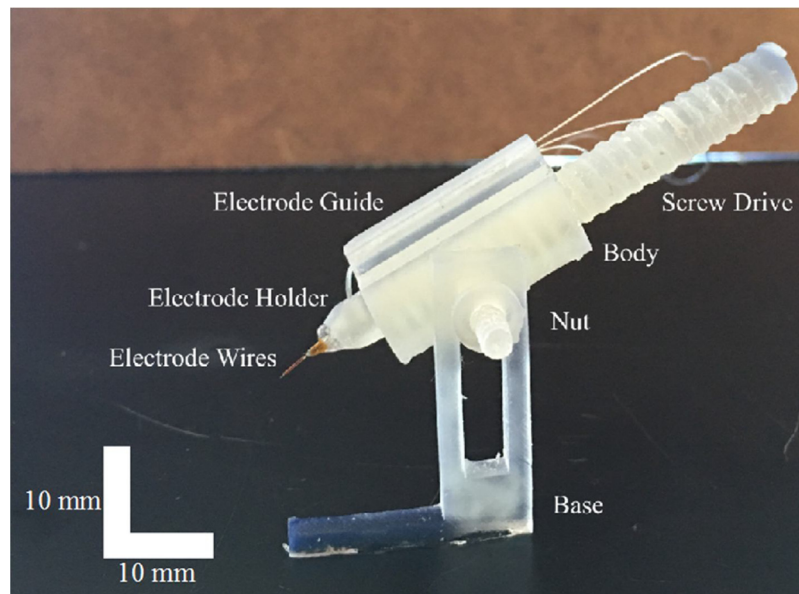


Fig. 3. Lateral view of the assembled micromanipulator with microwire electrodes inserted.



Fig. 4. Lateral (left) and dorsal (right) view of an implanted toadfish.

tronic Design Ltd., version 7), to discriminate individual units in the extracellular recording.

3. Results

Four 3D printed micromanipulators were fabricated and all were successfully implanted into four different toadfish (Fig. 5). In all implants, adjustment of the screw drive allowed recording from additional units after repositioning of the electrodes with units recruited up to 4 days following implantation. At least 4 units in each fish were individually identified based on waveform characteristics with up to 10 individual units identified in a single fish. The recording baseline remained steady during self-generated movement indicating that the implant was stable. The background noise from the recording electrodes remained constant throughout the experiment indicating no seawater intrusion into the device and post mortem examination confirmed that the seal remained intact and there was no water intrusion into the cranium. Fish behavior was consistent with previous chronic implants with the fish quiescent during the first 12–24 h after implantation as effects of the paralytic and anesthetic dissipate with respiration rates similar to controls with 24 h. Toadfish swimming movement was intermittent and limited to short distances (~30 cm) however dur-

ing spontaneous or prodded movements, normal behavior was observed.

4. Discussion

There have been few experiments in which neural activity has been recorded from freely moving fish. The majority of these studies used fixed electrodes that could not be repositioned. The first successful implant with a uniaxial manipulator using a single channel electrode was implanted in the toadfish. Forty-seven toadfish were needed to isolate 97 single units or approximately two per implant (Tricas and Highstein, 1990; Tricas and Highstein, 1991). The uniaxial manipulator was positioned using a micromanipulator and therefore may have been limited in what areas of the nerve were accessible. The advantage of the current micromanipulator is that the base is first affixed to the skull and both the vertical position of the drive and angle of the three electrode wires could be adjusted prior to final positioning. Additionally, multichannel recording is possible by differentially recording between various combinations of the three electrodes. Waveform discrimination was often able to isolate at least 2 and sometimes 3 units after each retraction and repositioning. While some may have been the same unit, it is likely based on previous studies and waveform analysis (Radford and Mensinger, 2014) that additional new units were being iso-

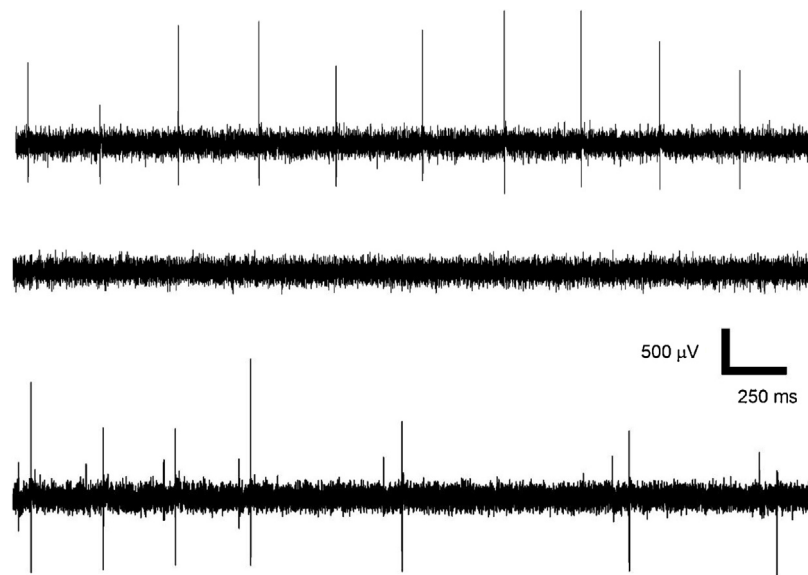


Fig. 5. The neural activity from an implanted fish. Top trace shows single unit activity, middle trace shows loss of activity after electrodes were withdrawn and bottom trace shows multiunit activity after electrodes were repositioned.

lated. The conservative estimate based on clearly distinct action potential waveforms was 4–10 units per implant.

The micromanipulator can be completely reused once the cyanoacrylate glue is removed from the body and the electrode replaced, however, the low cost of the material (<\$5 per print) and the short fabrication time of 2 h makes the entire assembly disposable. Alternatively, a new body and base can be printed as the screw drive and electrode holder can easily be reused following the removal of a small drop of cyanoacrylate glue from the electrode holder tip.

Continual access to the nerves over extended time periods is important for monitoring behavior but also has a number of additional uses. Seasonal plasticity could be observed inside and outside of the reproductive season. Short term (weeks to months) environmental changes or the effects of the sub lethal but chronic exposure to outside agents could be continuously monitored.

Three dimensional printing allows for rapid prototyping of custom designed devices to be formed at low prices. Prototypes can be quickly modified. The current micromanipulator was designed for the size of the adult toadfish available, but further miniaturization should be relatively straightforward to allow its use in smaller toadfish or other species. Future prototypes will incorporate a swivel base which will add an additional axis of rotation and greater flexibility. The goal of these initial implants was to test the feasibility of the equipment and waterproofness, and therefore recording was not tested past 4 days. However, the ability to reposition the electrodes has the potential to extend the recording period for weeks.

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