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Primary Cell Culture of Adult Zebrafish Spinal Neurons for Electrophysiological Studies

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Abstract

Background —Zebrafish (*Danio rerio*) are growing in popularity as a vertebrate model organism for the study of spinal neurocircuitry and locomotion. While many studies have used the zebrafish model system for electrophysiological analyses in embryonic and larval stages, there is a growing interest in studying spinal circuits and neurons from adult fish.

New Method —To expand upon the existing toolset available to the zebrafish research community, we have developed the first primary cell culture system of adult zebrafish spinal neurons. The intact spinal cord is dissected, and neurons are isolated through enzymatic digestion and mechanical dissociation. Identifiable neurons are viable for electrophysiological analyses after two days in culture.

Results —Spinal neurons in culture were confirmed by immunofluorescence labeling and found to exhibit distinct morphologies from other cell types, allowing neurons to be identified based on morphology alone. Neurons were suitable for calcium imaging and whole cell patch clamp recordings, which revealed excitable cells with voltage-gated whole cell currents, including tetrodotoxin-sensitive sodium currents.

Comparison with Existing Methods —This primary cell culture system is the only methodology available to isolate neurons from the adult zebrafish spinal cord. Other methods rely on keeping the spinal cord intact or the utilization of embryonic or larval stage fish. This method provides a robust platform for use in neurophysiological and pharmacological studies.

Conclusions —The novel primary cell culture system described here provides the first *in vitro* methodology available to isolate and culture neurons from the adult zebrafish spinal cord for use in electrophysiological analyses.

Graphical abstract

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Keywords

Adult Zebrafish; Spinal Cord; Electrophysiology; Cell Culture; Patch Clamp; Calcium Imaging

1. Introduction

As a genetic vertebrate model system, zebrafish (*Danio rerio*) have gained tremendous popularity among biologists from a variety of fields. In recent years, the zebrafish model system has been utilized for neurophysiological studies of neural circuits (Friedrich et al., 2010), including detailed studies of the spinal locomotory circuits of adults and larvae (Berg et al., 2018; Fetcho and McLean, 2010). In part, the popularity of zebrafish comes from its fully sequenced genome (Howe et al., 2013), rapid and well-documented development (Kimmel et al., 1995), and the creation of transgenic lines, thus allowing for highly selective fluorescent protein expression, calcium imaging and optogenetic studies (E. Brusteinstein et al., 2003; Fan et al., 2007; Kimura et al., 2013; McLean and Fetcho, 2011; Portugues et al., 2014; Sassen et al., 2017). The assortment of transgenic lines supports the already robust zebrafish model organism, making it a powerful vertebrate system for use in basic and biomedical research in the field of neuroscience.

One area where zebrafish have demonstrated their usefulness is in the study of the cellular, molecular, and physiological processes in the spinal cord (Fetcho et al., 2008; Fetcho and McLean, 2010; Lewis and Eisen, 2003). Most current studies utilizing the adult zebrafish have relied on *in vivo* and *ex vivo* preparations exclusively (Berg et al., 2018; Björnfors and El Manira, 2016), making use of the intact organism. Additionally, adult *in vitro* preparations have been used for such analyses, but current *in vitro* methodologies still rely on keeping the spinal cord intact (Gabriel et al., 2008), making it difficult to study neurons in the absence of neural connections. Embryonic and larval *in vitro* preparations have been used to study isolated neurons in culture (Andersen, 2001; Chen et al., 2013; Sakowski et al., 2012; Sassen et al., 2017; Tapanes-Castillo et al., 2014; Won et al., 2012), which is advantageous over *in vivo* preparations as they allow neurons to be studied in the absence of other neural inputs; however, embryonic spinal neurons are physiologically immature, lacking receptor systems that are often of great interest (Brusteinstein et al., 2003; McLean and Fetcho, 2004), therefore limiting the application of their use for electrophysiological analyses. A similar *in vitro* preparation using isolated spinal neurons from the adult zebrafish would be far more experimentally relevant for studying the spinal cord. However, to our knowledge, no such methodology has been developed.

To overcome these limitations, we have developed the first *in vitro* primary cell culture of adult zebrafish spinal neurons. Spinal neurons are isolated through enzymatic digestion of the dissected intact spinal cord from adult zebrafish and maintained through five days in primary cell culture for use in electrophysiological analyses. Resulting neurons in culture have distinct neuronal morphology from other cell types, as confirmed by immunofluorescence labeling, which allow them to be easily identified based on morphology alone. Neurons in culture have large cell somas, long extending neurites, and, as shown here, present a robust platform for neurophysiological studies including calcium imaging and whole cell patch clamp experiments.

2. Methods

2.1 Zebrafish

Adult (>6 months old), male and female, wild type zebrafish were used. Fish were obtained from Boston Children's Hospital and a local fish breeder. Fish were fed daily, experienced a 14/10-hour light/dark cycle, and were kept at 28.4°C. All protocols were approved and performed in accordance with the institutional animal care and use committee (IACUC) of Occidental College. Whenever possible, all efforts were taken to minimize animal suffering.

2.2 Laminin Coated Coverslip Preparation

Up to 12 coverslips were coated with 20 µl of 1–2 mg/ml laminin solution (Sigma-Aldrich) on ethanol dipped and flamed 5 mm glass coverslips (World Precision Instruments) using aseptic technique. After 30 minutes of incubation at room temperature, the laminin was recovered, and the glass coverslips were washed in Nanopure™ water before being dried by capillary action on sterile filter paper and stored in a parafilm wrapped polystyrene petri dish at 4°C for up to 1 week. Recovered laminin solution could be reused up to four times to prepare batches of coverslips.

2.3 Primary Cell Culture of Adult Zebrafish Spinal Neurons

Zebrafish were euthanized in an iced zebrafish system water bath prior to being decapitated. A slotted insert prevented direct contact with the ice in the bath. They were then transferred to extracellular recording solution (ECR; 134 mM NaCl, 2.9 mM KCl, 1.2 mM MgCl₂, 2.1 mM CaCl₂, 10 mM dextrose, and 10 mM Sodium HEPES at pH 7.4, adjusted with HCl) for dissection. The full, intact, spinal cord was dissected from the adult zebrafish, first by eviscerating and removing the skin, then exposing the vertebral column by removing the surrounding musculature, and carefully removing the individual vertebrae to expose the spinal cord. The dura surrounding the spinal cord was then lightly nicked using small Vannas spring scissors in between every other dorsal root to facilitate access for trypsinization, which was performed for approximately 35 minutes at room temperature in 0.25% trypsin solution (Thermo Fischer Scientific). The spinal cord was then moved into 300–600 µl of cell culture medium (47% Leibovitz's L-15 (Thermo Fischer Scientific), 0.47X Ringers, 4.69% heat-inactivated fetal bovine serum (FBS; Thermo Fischer Scientific), 93.90 units/ml penicillin-streptomycin (Thermo Fischer Scientific), and 0.01 mg/ml ofloxacin (Sigma-Aldrich)) and triturated using a 200 µl gel loading pipette tip for approximately 20–30 minutes or until the spinal cord was fully dissociated. Specific

attention was paid to areas lightly nicked prior to trypsinization for trituration, which facilitated mechanical dissociation of neurons from the spinal cord. The medium was equally distributed in 50 μ l volumes on the laminin-coated coverslips for 1 hour and 30 minutes while the cells adhered to the laminin. Cells were cultured at 28.4°C in 3 ml of cell culture medium with 0.5 nM of neurotrophin-3 (Sigma–Aldrich), a neuronal growth factor. The culture medium contained both serum and neurotrophin-3, both of which appeared necessary for healthy and identifiable neurons with extending neurites (data not shown). Cell culture medium was changed after the first 24 hours, and on day three in culture. The cells were optimal for electrophysiological analyses on days two through five in culture.

2.4 Immunofluorescence Microscopy

In order to characterize cells in culture as neurons, immunofluorescence labeling was utilized. Cells were fixed in 2% paraformaldehyde (Thermo Fischer Scientific) in 1X ECR overnight at 4°C. They were then permeabilized using phosphate buffered saline (PBS) and 0.5% Nonidet P-40 (Sigma-Aldrich) for 15 minutes before being blocked in PBS and 4% FBS at room temperature. Cells were then probed with a primary monoclonal mouse anti-acetylated tubulin antibody (Sigma-Aldrich), an axonal marker (Tapanes-Castillo et al., 2014), at a 1:5000 dilution and a monoclonal rabbit anti-NeuN antibody (Abcam), a neuronal nuclear marker (Gusel'nikova and Korzhevskiy, 2015), at a 1:100 dilution in PBS and 4% FBS overnight at 4°C. Cells were washed 5 times in PBS before being probed with a secondary goat anti-mouse Alexa Fluor 568 antibody (Thermo Fischer Scientific) at a 1:1000 dilution and a goat anti-rabbit Alexa Fluor 488 antibody (Thermo Fischer Scientific) at a 1:500 dilution in PBS and 4% FBS for two hours at 4°C.

Cells were then washed 10 times before being imaged using a Leica TCS SP5 II confocal microscope fitted with a 25X water immersion objective lens and recorded with LAS AF software (Leica). Cells in culture were imaged and their morphology was noted for later analysis using phase contrast microscopy.

2.5 Morphological Analysis

Neurons were imaged using a Nikon Eclipse TE2000-S inverted microscope equipped with phase contrast optics. Cells with neuronal morphology were characterized by large round somas, long and often highly branched extending neurites, and distinct circular phase contrast halos. Some neurons in culture were characterized by similar features, but also had dark and grainy cell somas and blebbing axons, which were found not to be viable for electrophysiological analyses. Other cell types in culture were often of glial morphology, which was characterized by small cell bodies, tapering processes, and large, and in some cases, long cellular extensions. To examine extending neurites, cells were prepared for scanning electron microscopy (SEM) in 3% glutaraldehyde (Ted Pella) in a SEM buffer solution (0.1M sodium-phosphate, 0.1M sucrose, at a pH of 7.4 adjusted with HCl) overnight at 4°C. They were then washed and dehydrated in steps of 35%, 50%, 65%, 80%, 95%, and 100% ethanol. Cells were then placed into hexamethyldisilazane (HMDS; Ted Pella) until evaporated. Coverslips were mounted on pin stubs using an adhesive carbon conductive tab (ELCO Tabs™; Ted Pella), sputter coated, and imaged using the FEI Phenom

scanning electron microscope. To highlight the growth cones of extending neurites SEM images were false colored using Adobe Photoshop (Version 19.1.4).

2.6 Calcium Imaging

Cells on days two through five in culture were loaded with 5 μ M Oregon Green 488 Bapta-1, AM, calcium sensitive dye (Thermo Fischer Scientific) prepared in DMSO (0.1%) for a minimum of 20 minutes. Coverslips were then moved to 1X ECR before being imaged on the confocal microscope, as described above. Images were acquired every 537 ms throughout solution exchange. Resting levels of calcium were imaged. To induce calcium responses, a gravity fed solution was exchanged with a modified ECR containing elevated potassium (High K⁺ ECR; 74 mM NaCl, 81.2 mM KCl, 1.2 mM MgCl₂, 2.1 mM CaCl₂, 10 mM dextrose, and 10 mM Sodium HEPES at pH 7.4, adjusted with HCl). In addition, responsive neurons were repolarized by solution exchange back to ECR. Fluorescence emission was monitored throughout solution exchanges and average fluorescence levels were analyzed using the Time Series Analyzer V3 plugin (<https://imagej.nih.gov/ij/plugins/time-series.html>) in ImageJ software. False coloring was performed on adjusted images using the Fire LUT in ImageJ. Offline time subtraction was performed in Microsoft Excel (Version 16.17) and data was graphed in Igor Pro (Wavemetrics; Version 8.02). Maximal and recovered responses were determined and used to calculate means and standard errors of responses to depolarizing and repolarizing conditions.

2.7 Patch Clamp Recordings

Whole cell patch clamp recordings were performed on days two through five in culture. Patch pipettes were pulled from 1.5 mm patch clamp glass (#8250; World Precision Instruments) using the HEKA PIP5 pipette puller and sylgarded on the taper (Sylgard® 184; World Precision Instruments) before being heat polished to 4–9 M Ω . The pipette was filled with intracellular recording solution (ICR, 125 mM K-gluconate, 2.5 mM MgCl₂, 10 mM EGTA, 4mM ATP, 10 mM Potassium HEPES, at pH 7.4, adjusted with KOH). Recordings were acquired with the HEKA EPC-10 amplifier (gain set between 2–10 mV/pA) and filtered with two Bessel filters (10 KHz and 2.9 KHz). Cells were visualized using a Nikon Eclipse TE2000-S inverted microscope and whole cell patch clamping was performed using the HEKA EPC-10 head stage and a Sutter MP-225 micromanipulator. Whole cell patch-clamp recordings were carried out on neurons in 1X ECR bath solution. Standard IV protocols with P/4 subtraction were used to collect voltage clamp data with series resistance compensation (10 μ s; 60–80%). Neurons were held at –80 mV in the whole cell configuration with voltage steps between –60 mV and +50 mV. Offline leak subtraction was performed in Microsoft Excel and data was graphed and analyzed in Igor Pro. Bridge-balanced current clamp recordings were also acquired following the establishment of the whole cell configuration in voltage-clamp mode. To identify tetrodotoxin (TTX) sensitive sodium currents in these neurons, whole cell patch clamp recordings were performed on neurons in a bath application of 460 nM TTX. Standard IV protocols with P/4 subtraction were carried out, as described above, before and after application of TTX. Currents were then subtracted using Microsoft Excel, then plotted and analyzed in Igor Pro.

3. Results

3.1 Primary Cell Culture of Adult Zebrafish Spinal Neurons

To determine if neurons from the spinal cord of adult zebrafish could be recovered in primary cell culture, fully intact spinal cords were dissected from freshly euthanized fish. The dura of the spinal cord was lightly nicked using small Vannas spring scissors to facilitate enzyme access during trypsinization. Trypsinization of 15, 20, 25, 30, 35, and 40 minutes were tested for the efficient recovery of viable cells. Spinal cords trypsinized for 35 minutes could be easily triturated, yielding consistently high cell counts (14–41 viable neurons per coverslip), robust neurite outgrowth, and stable patch clamp recordings.

Trituration of the trypsinized spinal cord was performed to dissociate the neurons from the spinal cord into solution. Two methods were used for trituration, one where trituration was performed in a larger volume of cell culture medium, followed by low-speed centrifugation and resuspension, and another where the spinal cord was triturated in the final volume required for direct plating on coverslips. Preparations where the spinal cord was triturated in the final volume used for cell plating not only yielded consistently higher cell counts (14–41 viable neurons versus 9–21 per coverslip), but also produced better, more robust cells for electrophysiological analyses. Therefore, the steps involving centrifugation and resuspension were eliminated.

For ease of transfer for neurophysiological recordings and calcium imaging, cells from the dissociated spinal cords were plated on 5 mm round coverslips. Cells did not adhere to the untreated glass coverslips, so to facilitate cell adhesion poly-D-lysine (0.01%, Sigma-Aldrich), poly-D-lysine + laminin (Sigma-Aldrich), or laminin alone (1–2 mg/ml) coating of coverslips was tested. While coverslips surface coated in either combination containing Poly-D-lysine resulted in excess cellular debris and low viable cell recovery, laminin alone resulted in minimal debris adhesion while successfully promoting neuronal adherence and neurite outgrowth (data not shown). Therefore, all experiments in this study utilized laminin coating to support cellular adhesion.

Cells were cultured in medium containing the pH indicator phenol red. Change in the color of cell culture medium was observed around 24-hours after plating, indicating mild acidification. Therefore, cell culture medium was changed at that time. Additionally, the same observation was seen 48-hours thereafter and the culture medium was again changed at day 3. Non-adherent debris decreased considerably with each medium change (data not shown). Initially, cells were incubated at room temperature, which yielded moderate cell counts (5–16 viable neurons per coverslip), but produced cells inconsistent for electrophysiological analyses. Cells incubated at room temperature were sometimes fragile and easily lysed when used for patch clamp studies. Upon incubating at 28.4°C, neurons were higher in density, had larger and farther extending neurites, and were far better for use in electrophysiological studies. Similar cell counts (14–41 viable neurons per coverslip) were also observed for plating up to 12 coverslips from the same volume of dissociation medium, thus indicating that there may be a cell density dependent component to their recovery and maintenance in primary cell culture.

3.2 Neural Identification and Morphological Analysis

To confirm the presence of neurons among the cells isolated from the adult zebrafish spinal cord, as well as to establish criteria to distinguish neurons from other cell types based on morphology, immunofluorescence microscopy was performed using antibodies to acetylated tubulin and NeuN (neuronal nuclear protein). Anti-acetylated tubulin labels acetylated alphas-tubulin, an axonal marker for neurons (Tapanes-Castillo et al., 2014), while anti-NeuN marks the nuclei and perinuclear cytoplasm of neurons (Gusel'nikova and Korzhevskiy, 2015). Cells in culture that positively labeled for both acetylated tubulin and NeuN were classified as neurons (Figure 1b, c, d), which had distinct morphology from other cell types under differential interference contrast (DIC) imaging (Figure 1a, e, f, g, h, and further described below). Not all neurons that positively labeled for NeuN showed acetylated tubulin labeling on all extending neurites, indicating that neurons in culture have a mix of different branching patterns of axons and dendrites (data not shown).

Under phase contrast imaging, neurons were further characterized. Neurons in culture had large circular cell somas, long extending neurites with unique branching patterns, and a distinct circular phase contrast halo around their cell somas (Figure 2a, b, c). Extending neurites were highly active, as seen during time lapse recordings (Supplemental Video 1). SEM images of neurites extending from neurons in culture provided a snapshot of growth cones with highly branched filopodia (Figure 2d). Not all neurons in culture seemed viable for analysis, likely due to unsuccessfully surviving the enzymatic digestion and dissociation process (0–11 neurons per coverslip). Neurons that were not viable for analysis had neuronal morphology, but dark and grainy cell somas and often blebbing axons (Figure 2e).

Other cell types in culture that did not label for acetylated tubulin and NeuN also had distinct morphology that made them easily differentiable from neurons in culture (Figure 1; 2–19 other cell types per coverslip). Although we did not characterize these cell types, we assume they are likely glial, as the zebrafish spinal cord is known to contain oligodendrocytes (Park et al., 2002; Tomizawa et al., 2000a), astrocytes (Kawai et al., 2001), and radial glial cells (Tomizawa et al., 2000b). These other cell types had smaller cell soma that were often oblong in shape, as well as tapering or, in some cases, large cellular extensions, similar, but distinct from neurites (Figure 2f).

3.4 Calcium Imaging

In order to test the viability of using isolated neurons from the adult zebrafish spinal cord for more detailed electrophysiological analyses, calcium imaging was performed. Neurons loaded with 5 μ M Oregon Green 488 Bapta-1, AM, calcium sensitive dye had low levels of fluorescence in normal 1X ECR followed by robust increases in fluorescence emission in response to solution exchange with high K^+ substituted ECR (Figure 3a). Consistent changes in fluorescence intensity, indicating an increase in calcium concentration inside the cell, were observed in the neurons tested as a rapid response to solution exchange (Figure 3b). In cells tested, an average maximal change in fluorescence (F/F) of 1.07 (SEM $\pm$ 0.02; $n=5$) was observed (Figure 3c). In order to test whether neurons in culture could recover following depolarization in high K^+ substituted ECR, depolarized neurons were returned to normal ECR, resulting in recovery from elevated calcium (Supplemental Figure 1a, b).

Additionally, depolarization and recovery could be performed multiple times on single neurons (Supplemental Figure 1c). These data establish the functional utility that these adult spinal neurons can be used for calcium imaging studies as well as the likely presence of voltage-gated ion channels to be studied with electrophysiological approaches.

3.5 Whole Cell Patch Clamp Recordings

Cultured neurons on days two through four post-isolation were utilized for whole cell patch clamp recordings. Whole cell currents recorded under voltage clamp revealed the presence of both rapidly activating and inactivating inward currents characteristic of sodium channels, as well as rapidly activating outward currents characteristic of the delayed-rectifier voltage-gated potassium channels (Hille, 2001; Figure 4a). Current-voltage relationships (IV plots) of the series resistance corrected recordings show inward currents activating around -40 to -30 mV and outward currents activating at more depolarized voltages between -30 and -20 mV (Figure 4b, c). In neurons with larger peak inward currents (>500 pA) action potentials could be elicited in the adult spinal neurons with depolarizing steps in current clamp mode (Figure 4c). Single spiking was observed followed by membrane oscillations. Tetrodotoxin sensitive sodium subtraction currents were also determined by comparing whole cell current traces before and after the addition of 460 nM TTX to the bath solution (Figure 4d). TTX-resistant inward currents were not noted, as all of the voltage-dependent inward currents were blocked at this concentration. This initial characterization of the electrical properties of the cultured adult zebrafish neurons demonstrates the utility of this preparation for a diverse set of electrophysiological studies, including neuronal excitability and neurotoxins research.

4. Discussion

Here, we introduce a novel *in vitro* primary cell culture system utilizing the adult zebrafish spinal cord as a source of central nervous system neurons for electrophysiological studies. Our robust platform utilizing the zebrafish model system improves upon the limited methodology available to scientific communities studying spinal circuitry and locomotion. Isolating viable neurons from adult vertebrates is inherently difficult (Eide and McMurray, 2005); however, their use in neurophysiological analyses has advantages over the immature embryonic and larval preparations. Our *in vitro* preparation allows neurons to be isolated in culture from the adult zebrafish, making them not only accessible for electrophysiological analyses, but also for other interrogations that require neurons to be in isolation from neuronal inputs, as difficult in *in vivo* and *in situ* preparations. This novel adult preparation adds to the growing arsenal of approaches available to scientists utilizing the powerful zebrafish model system.

Our culture system supports cell adherence and neurite outgrowth following enzymatic and mechanical dissociation from the dissected intact spinal cord. Cells in culture that positively label for acetylated tubulin, an axonal indicator, and NeuN, a neuronal marker, have distinct morphology from other cell types in culture, allowing them to be easily identified for experimentation based on morphology alone (Figures 1 and 2). For experiments that use methodologies including but not limited to patch clamping and calcium imaging, this is

extremely beneficial as neurons must be discriminated from other cell types in culture prior to experimentation. For neurophysiological experiments on general neuronal populations, additional labeling is not necessary, as neurons are easily identified. However, additional labeling can be used for experiments that involve specific neuron populations.

For experiments that require further characterization of specific neuronal populations, the primary cell culture system described here could exploit the ever-expanding zebrafish model system's variety of transgenic lines. Many transgenic lines offer cell type specific fluorescent protein expression, including beyond the larval stages, making this a robust platform for many areas of study (Song et al., 2018, 2016). Examples of cell selective expression include GFP expression as a reporter of olig2 (Shin et al., 2003), which labels oligodendrocytes and spinal neurons, alx (Kimura et al., 2006), a homolog of chx10, which labels excitatory interneurons in the spinal cord, and even neurons with expression of specific neurotransmitters, such as dopaminergic neurons in the ventral diencephalon (Xi et al., 2011). During experimentation, neurons could be identified not only based on morphology, but also by the expression of the reporter genetically encoded in the zebrafish line. This would allow neurons of particular interest to be identified prior to experimentation and allow for detailed studies on specific neuronal populations.

In addition to isolating adult zebrafish spinal neurons in primary culture, we demonstrate their utility for functional physiological studies, including calcium imaging on days two through five in primary cell culture. Calcium imaging is a widely used tool for neurophysiological studies in the zebrafish model system (Kettunen, 2012). We show that neurons in culture can be conveniently and noninvasively loaded with the acetoxymethyl ester of the calcium sensitive dye Oregon Green 488 Bapta-1. These neurons exhibit robust responses to depolarization with elevated potassium in the external solution (Figure 3). Additionally, they recover in response to solution exchange back to ECR (Supplemental Figure 1). Although the magnitude of the observed responses likely represents an upper limit for these neurons, the strength of the responses suggests that calcium imaging with this preparation will be widely applicable.

Our novel primary cell culture system also offers a robust platform for scientists looking to generate whole cell patch clamp recordings as part of detailed neurophysiological analyses. Here, we show that neurons on days two through four in culture can be utilized for whole cell voltage and current clamp recordings. Whole cell recordings revealed inward and outward voltage-dependent currents typical of neurons and similar to those recorded in other zebrafish *in vitro* and *in situ* preparations (Pineda et al., 2005; Won et al., 2012). Isolated TTX-sensitive subtraction currents also demonstrated that the inward currents are predominately facilitated by TTX-sensitive voltage gated sodium channels in the neurons tested. TTX resistant inward currents were not observed, suggesting the absence of these currents, as has been observed in the Rohon-Beard sensory neurons of larval zebrafish (Pineda et al., 2005).

Based on our experiments, we have observed that neurons on days two through five in culture are best suited for neurophysiological experimentation. While cells extending neurites can be detected by day one in culture, suitable numbers of neurons for recordings

are present by day two, which can be identified based on the morphological criteria supported by immunofluorescence localization of acetylated tubulin and NeuN. The observation of stem/progenitor cells in the primary cell cultures from the zebrafish brainstem suggests the possibility that neurons in our cultures could have differentiated during or after plating (Tapanes-Castillo et al., 2014). While the neurons on day two have similar current amplitudes and voltage-dependent current kinetics to those on days three and four (data not shown), the presence of progenitor cells directly differentiating into neurons during plating cannot be ruled out. Starting on day five, the elaboration of neurites and associated increase in cell capacitance make proper space clamp and series resistance compensation difficult for patch clamp studies. While difficult for patch clamp studies this preparation could be utilized past day five for other types of experiments, including calcium imaging studies.

Our primary cell culture of adult zebrafish spinal neurons provides a novel tool and avenue for using the adult zebrafish in neurophysiological studies. Other methodologies that have utilized the zebrafish model system have generated reasonable platforms for analyses of the spinal cord, but isolated adult spinal neurons offer specific advantages over the embryonic and larval preparations, as the neurons in culture are from a physiologically mature source, have larger cell somas, and have proven robust for electrophysiological analyses. Many potential studies, including but not limited to studying the neurocircuitry of the spinal cord and locomotion (Berg et al., 2018), can utilize this primary cell culture system. Further characterization of the neuronal populations in the zebrafish spinal cord is of great interest (McLean and Fetcho, 2008), and this preparation offers a novel tool for such studies. The ability of the zebrafish to regenerate axons within the central nervous system has been studied extensively as well (Bhatt et al., 2004; Graciarena et al., 2014; Reimer et al., 2008). In addition to its use in electrophysiological studies, this system could provide a platform for researchers to observe axonal regeneration of spinal neurons completely isolated from other direct neuronal and glial contacts, which would offer a unique preparation to complement existing studies. Finally, this system adds to the growing number of neuronal primary cell culture systems now available from embryos to adult zebrafish (Andersen, 2001; Chen et al., 2013; Sakowski et al., 2012; Sassen et al., 2017; Tapanes-Castillo et al., 2014; Won et al., 2012).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations –

DIC	differential interference contrast
ECR	extracellular recording solution
FBS	fetal bovine serum
HMDS	hexamethyldisilazane
IACUC	Institutional Animal Care and Use Committee
ICR	intracellular recording solution
IV	current voltage
NeuN	neuronal nuclei
PBS	phosphate buffered saline
SEM	scanning electron microscopy

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Highlights

- A method for isolating spinal neurons from adult zebrafish spinal cords is described.
- The neurons in primary cell culture can be utilized for calcium imaging and whole cell patch clamping.
- The cultured neurons are a novel tool for studying isolated spinal neurons from adult zebrafish.

Figure 1 –. Cells in Culture Positively Label for Neuronal Indicators

Cells isolated from the adult zebrafish spinal cord in primary cell culture for four days were fixed, permeabilized, and probed with anti-acetylated tubulin (AcTub; b, f) and anti-NeuN (c, g) antibodies. Cells that positively label for both AcTub and NeuN (b, c) have distinct morphologies (a) from other cell types (e) in culture. Composite images show distinct labeling of AcTub on neurites and NeuN in the cell soma of the neuron (d), but not the other cell type (h). (Scale bars: neuron, 15 μ m; other, 25 μ m).

Figure 2 –. Neurons in Culture are Identifiable Based on Morphology Alone

Neurons at day four in primary cell culture are easily distinguishable from other cell types under phase-contrast microscopy. a, b, c. Neurons in culture have large round cell somas with distinct phase-contrast halos. Neurons also have long extending neurites that, in some cases, were highly branched. d. SEM of growth cones on extending neurites reveal highly branched and detailed processes. e. Neurons characterized by dark and grainy cell somas and blebbing axons (top neuron) are easily distinguishable from neurons better suited for electrophysiological analyses (bottom neuron). f. Other cell types in culture were also

observed, which were characterized by smaller cell soma and long tapering processes distinct from neurons. (Scale bars: a, b, c, e, f, 20 μm ; d, 5 μm).

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Figure 3 –. Calcium Imaging Reveals Neurons are Suitable for Electrophysiological Studies
 Neurons at day 4 in primary cell culture were loaded with 5 μ M Oregon Green 488 Bapta-1, AM, dye. Cells were imaged in 1X ECR followed by solution exchange into High K⁺ substituted ECR solution. a. False-colored cell soma responding to high K⁺ ECR. b. Large amplitude changes in fluorescence were observed in cells tested directly after solution exchange (n=5; each color indicates one trial). c. Mean maximal change in somal fluorescence was 1.07 (SE $\pm$ 0.02; n=5). (Scale bars: 5 μ m).

Figure 4 –. Whole Cell Patch Clamp Recording of Neurons in Culture

Whole cell patch clamp recordings on neurons at day two through four of primary cell culture were performed. a. Example whole cell currents evoked by standard IV protocols in 12 step increments (10 mV steps from -60 to +50 mV). b. IV relationship of mean peak inward currents of series resistance corrected recordings (n=9; error bars are SE). c. IV relationship of mean peak outward currents of series resistance corrected recordings (n=9; error bars are SE). d. Example current clamp recordings eliciting action potentials (10, 40,

100 pA steps; 50 ms). e. TTX subtraction currents from pre and post 460 nM TTX addition (20 mV steps from -50 to $+30$).

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