



Preparation of dissociated Zebrafish spinal neuron cultures

Søren S. L. Andersen

University of California at Berkeley, Department of Molecular and Cellular Biology, 142 Life Sciences Addition
#3200, Berkeley, CA 94720-3200, USA

Accepted in revised form 28 November 2001

Abstract. The method described here explains a simple protocol for how to prepare dissociated Zebrafish spinal neuron cultures. The neurons grow fast in a simple culture medium and at room temperature. Considering the advantages afforded by

the optical transparency of the Zebrafish embryo combined with the powerful molecular perturbation techniques available, this technique has potential to further advance molecular analysis of axon growth and guidance.

Key words: growth, guidance, *in vitro*, neuron, *Xenopus*, Zebrafish

1. Introduction

The ability to dissociate nervous tissue and culture individual neurons *in vitro* has been essential to reach our present understanding of how the nervous system functions and propagates signals. The oldest of these culture systems is Harrison's *Xenopus* spinal explant culture system [1]. The neuronal culture systems mostly used today are based on vertebrate models like rat, mouse, chick or *Xenopus*. These systems are very useful for a number of questions, but have several drawbacks. These vertebrates cannot be subjected to forward genetic approaches. There is no genome sequencing project for these vertebrates. Neuronal cultures prepared from these animals need to grow at 37 °C (with the exception of *Xenopus*), and observation of growing neurons *in vivo* is difficult. The equipment and facilities required are costly.

In recent years, *Xenopus* has been used extensively as a model system to study growth cone/axon guidance in an extracellularly applied gradient [2, 3]. One reason for this is that mammalian neurons are more delicate to handle than *Xenopus* neurons, and the latter grow at room temperature. Moreover, *Xenopus* neurons grow fast at room temperature and have big neurites/growth cones, which is an advantage for cell biology related questions. *Xenopus* has the big disadvantage that molecular perturbation experiments, like transfections, viral infections and knock-out experiments, are either not available or difficult. To molecularly perturb a *Xenopus* neuron, one has to microinject the *Xenopus* embryo with protein, RNA or DNA shortly after fertilization. These procedures are labor intensive, preclude studies of molecules essential for cell division and one has no control over the expression level of the proteins studied.

It would be useful if neuronal *in vitro* systems based on genetically powerful organism like *Drosophila melanogaster* or *Caenorhabditis elegans* were available. It has been attempted to make *C. elegans* cultures but the neurons are very small and do not grow well [see http://www.dartmouth.edu/artsci/bio/ambros/protocols/worm_protocols.html]. *Drosophila* cultures have been produced with higher success than *C. elegans* cultures [4, 5]. However, the *Drosophila* cultures also suffer from the small size of the neurons, are difficult to reproducibly make and the neurons do not grow well.

It is in the light of the above considerations that I sought to develop an easy vertebrate neuronal culture system amenable to genetic manipulation, with neurons of sufficient size to be observed at moderate magnification *in vitro* as well as with possibilities of *in vivo* observation. As model organism, I chose the Zebrafish, *Danio reio*. Zebrafish have a generation time of only about 2–3 months. Development occurs externally and the embryo is optically transparent which has allowed investigators to observe growing neurons in the intact embryo [6–11]. Previous papers have reported on neuronal cultures prepared from whole Zebrafish embryos or from Zebrafish retina [7, 12, 13]. In contrast, this method aims at describing how to easily and rapidly prepare dissociated Zebrafish spinal neuron cultures with the specific aim at observing and measuring the growth of neurites. The method described was inspired by the technique used to prepare dissociated *Xenopus* spinal neuron cultures [14–16]. The *Xenopus* system has in the past decade brought significant advances to our knowledge of how a growth cone senses its environment. This Zebrafish culture system provides an additional tool to extend the analysis of axon guidance and neurite growth.

2. Materials

Chemicals were from Sigma, Boehringer-Mannheim or Gibco BRL, and culture dishes from Falcon (cat. No. 351008; 35 × 10 mm). Custom ATV solution was from Irvine Scientific, CA (cat. number 9920). 24 × 40 mm cover glass was from Fisherbrand VWR. Fetal bovine serum was from Invitrogen (cat. number 10100-139). Laminin was from Sigma (cat. number L-2020).

3. Procedures

A. Collection and development of Zebrafish embryos

Ten to twenty wild-type Zebrafish embryos were collected from the aquarium within two hours of fertilization. They were then washed with 0.1% bleach and transferred to embryo medium, as described [8]. Embryos were allowed to develop overnight in embryo medium at 23–25 °C to moderate the rate of development (instead of the optimal 28.5 °C). After approximately 24 hours at 23–25 °C the embryos had reached the 18-somite stage, and were collected for culture preparation (axons of motor neurons appear *in vivo* at the 18-somite stage [6]).

B. Isolation and dissociation of spinal cords

The 18-somite stage embryos were transferred with a Pasteur pipette to a 70% ethanol solution for disinfection. After about 5 seconds in the 70% ethanol they were washed with MMR and then transferred to fresh MMR for dissection (MMR (in mM): 100 NaCl, 2 KCl, 1 MgSO₄, 2 CaCl₂, 5 Na-HEPES, 0.1 EDTA; pH = 7.8). The chorion of the embryo was removed with forceps under a stereomicroscope. Subsequently the spinal cords were isolated by removing the yolk bag, and then the head as well as part of the spinal cord (Figure 1). For dissociation [8], the spinal cords were transferred with a Pasteur pipette from the MMR solution to Custom ATV solution (Custom ATV (in mM): 0.6 EDTA, 5.5 Glucose, 5.4 KCl, 136.8 NaCl, 5.5 Na₂CO₃, 0.05% w/v Trypsin; stored in 10 ml aliquots at –20 °C), and incubated for 10–15 min at room temperature. After about 10–15 min in ATV solution, the spinal cord tissue had partially dissociated. To fully dissociate and plate the tissue, two spinal cords/cover glass were taken up with a drawn-out Pasteur pipette having an opening about the diameter of a spinal cord. The fully dissociated spinal cords were then plated.

C. Plating of dissociated spinal cords

The dissociated spinal cords were plated in streaks on 24 × 40 mm cover glass (Fisherbrand VWR) in a petridish (Falcon cat. No 351008; 35 × 10 mm) with medium consisting of: 50% L-15 (GIBCO BRL), 49% 10-fold diluted Ringer

(Ringer (in mM): 115 NaCl, 2.6 KCl, 2 CaCl₂, 10 Hepes; pH = 7.6) and 1% fetal bovine serum [15]. Prior to plating of the dissociated spinal cords, the glass was sterilized with 100% ethanol. After plating, care was taken not to move the dishes as this prevents adhesion of cells to the glass. As the dissociated tissue is transparent, plating is facilitated using a black background. The cultures were left to develop at room temperature (21 °C–24 °C). While growth of neurons plated under these conditions were observed, dramatic improvements were obtained if the glass was precoated with Laminin. All studies reported here were performed using Laminin coated glass. To make Laminin coated glass, a solution containing 1 mg Laminin (Sigma Chemicals, catalogue number L-2020) was aliquoted by 50 µl and stored at –80 °C until use. Prior to Laminin coating, one 50 µl Laminin aliquot was gently diluted with 1 ml PBS. 250 µl of this solution was added onto the cover glass and incubated over-night at 4 °C. Prior to culture plating, the Laminin coated glass was washed once with PBS and twice with culture medium.

D. Observation and measurement of neurite growth

Recording of growth rates was performed at room temperature from 6 to 8 hours after plating using an inverted microscope equipped with a 20× lens and a CCD camera. With an approximately 15 minutes interval, the culture dishes were briefly illuminated for recordings. Recordings were directly into a MacIntosh G3 PowerBook through an Interview XLR8 digitizer with the accompanying Strata Videoshop software (XLR8, GA,

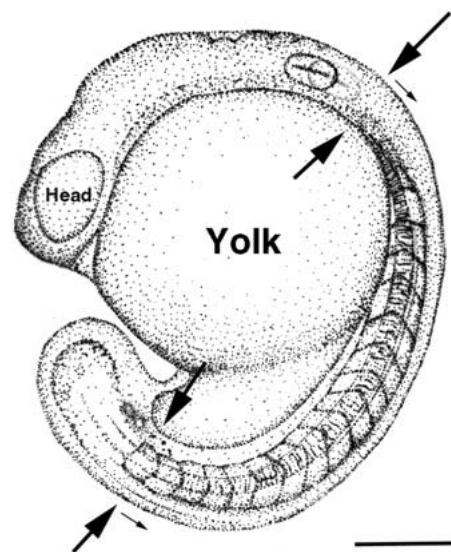


Figure 1. Drawing showing the morphology of a Zebrafish embryo at the 18-somite stage and after removal of the chorion. Prior to dissociation, the large yolk bag was freed from the spine. The large arrows indicate where the head and the spine were snapped off with forceps. Small arrows outline the portion of the spinal cord that was used for culture preparation. Bar represents 250 µm.

USA). Measurements of neurite length increases over time were performed using the NIH 1.62 program (Strata Videoshop movies open directly with the NIH program). For each neurite, the measurements were pasted from the NIH program into Microsoft Excel. An average growth rate for each neurite was estimated as the slope of the line that best fit the data. The average growth rate of all neurons was calculated as the average of the slopes of the individual neurites.

4. Results and discussion

The aim of the experiments described here was to develop a preparation of dissociated Zebrafish spinal neurons that would permit their growth *in vitro* to be analyzed in real time. Zebrafish embryos at the 18-somite stage were used for culture preparation because axons of motor neurons appear *in vivo* at the 18-somite stage [6]. For practical reasons, the embryos developed to the 18-somite stage at 23–25 °C instead of at the optimal 28.5 °C. Following dechoriation, the yolk and the head was manually removed with forceps. Part of the spinal cord was also removed leaving only its central part (Figure 1). While this method of spinal cord isolation is labor intensive, it ensures purity and reproducibility of the culture preparation. The isolated spinal cords were then dissociated. Dissociation using mild conditions like a Calcium-Magnesium free Ringer solution was insufficient to dissociate the spinal cord tissue, and it was found necessary to use a Trypsin containing solution. It was discovered that the development at 23–25 °C not only retarded embryo development, but also facilitated dissociation of spinal cord tissue, compared to embryos developed at the optimal 28.5 °C. After dissociation, the dissociated spinal cord tissue was from a Pasteur pipette applied in streaks onto a coverglass located in a medium-containing petridish. Several media were tested. Of the media tested, the one that best supported growth of neurons contained 50% Leibovitz-15 and 1% fetal bovine serum and a buffer. The number of neurons as well as their growth rate increased significantly if the cultures were plated on Laminin coated glass. Therefore, all experiments were performed using Laminin coated glass. It was found that the development and growth of neurons occurred at room temperature and did not require 28.5 °C. The viability of the cultures beyond the end of the growth-phase of the neurons was not quantified as the goal of these experiments was to develop an assay for the acute observation of freshly prepared neurons.

6 to 8 hours after plating, the cultures were observed on an inverted microscope with a 20× objective. A heterogeneous population of cells were observed. There were some flat extended cells that,

based on their morphology, were probably either epithelial or fibroblast cells (as previously described [17]), some spherical cells that were probably myocytes and also neurons (data not shown). The number of neurons (prepared from two spinal cords) was variable and varied from a few up to about 50/cover glass. While the majority of cells observed were non-neuronal cells, the plating technique ensures that freely growing/isolated neurons are always encountered. Both monopolar and bipolar neurons were observed, and multiple neuronal spinal subtypes have been identified *in vivo* [18]. For this study, I chose to characterize the average growth rate of bipolar neurons (Figure 2). To this end, the growth of the neurons was observed for 30 to 90 minutes with an interval of approximately 15 minutes. Cultures made on five different days were used to estimate the average growth rate. Out of 14 bipolar neurons, with a total of 28 neurites, 22 neurites were observed growing. The length of the neurites versus time was plotted. As the growth rate of the neurites appeared to be fairly constant, an average growth rate for each neurite was estimated as the slope of the line that best fit the data. The calculated average growth rate was 35.8 μm/h + 18.2 μm/h (n = 22). This growth rate is twice that of dissociated *Xenopus* spinal neurites which grow at about 15 μm/h on plain glass ([2]; a study of the growth rate on Laminin has not been reported). These bipolar neurons have a shaft and a growth cone that are approximately 2–5 times smaller than the *Xenopus* neurons routinely used in growth cone turning experiments (data not shown).

The method described has several advantages that should make it an attractive alternative and supplement to already existing systems like *Xenopus* dissociated spinal neuron cultures. It is easier to reproducibly prepare good Zebrafish cultures than to reproducibly prepare the equivalent *Xenopus* cultures. Thus, whereas the viability of the neurons in *Xenopus* cultures is rather variable one usually does not have this problem with the Zebrafish cultures. If the Zebrafish embryos are of decent quality/appearance one usually gets good cultures which is not the case with the equivalent *Xenopus* cultures. The Zebrafish neurons grow fast, at room temperature and have sufficient size to be observed with low cost microscope equipment. The genetic and molecular techniques available with Zebrafish combined with its optical transparency and this culture system, offer a number of interesting experimental possibilities.

The establishment of genetic screens is possible, many mutants are already available, and the genome will supposedly be fully sequenced in the year 2002 [10]. While techniques for making knock-in or knock-out Zebrafish strains do not yet exist, such may become available in the future [19]. Moreover, recent studies have shown that new RNA antisense ‘knockout’ techniques work very well in all tissues

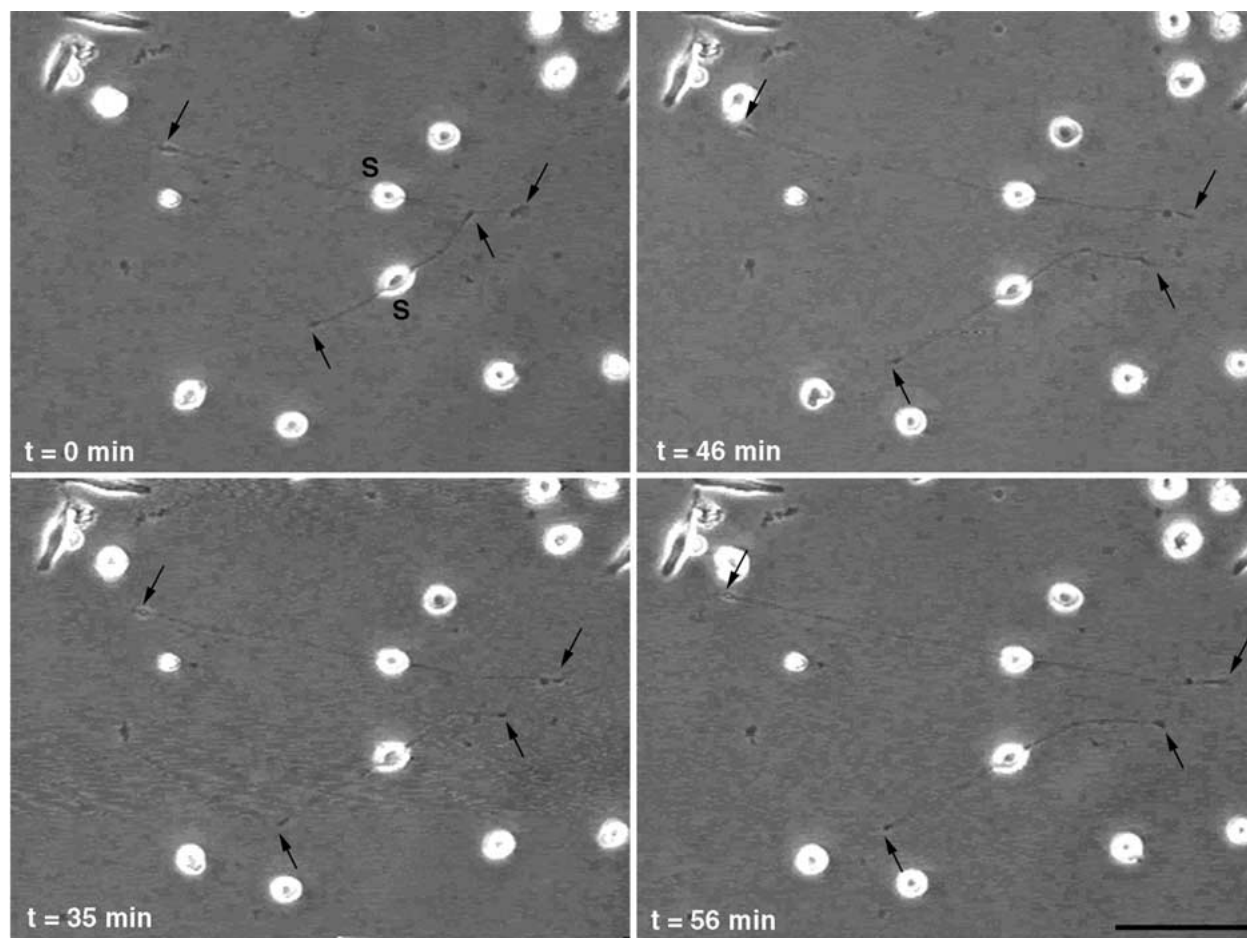


Figure 2. Time course of the growth of representative bipolar neurons. S is soma and arrows point to the growth cones. Time in left corner is in minutes. Bar represents 70 μm .

of the Zebrafish [20], and a large body of EST information already is available (<http://zfish.uoregon.edu>). One can imagine a heterologous culture system for electrophysiology recordings consisting of dissociated Zebrafish (wild-type and/or mutant fish) and dissociated *Xenopus* components, as has been achieved using *Xenopus* and *Drosophila* [5]. With such a heterologous culture system, it might be possible to specifically knock out proteins on either the postsynaptic or the presynaptic side of the neuromuscular junction in order to study the function of such proteins.

The primary use of the culture system described here will be to extend the analysis of axon growth and pathfinding. The optical transparency of the embryo has allowed the observation of axon pathfinding in the Zebrafish spinal cord *in vivo* (for example references 6, 7 and 9). With the present culture system it is possible to extend this analysis and molecularly perturb spinal neurons *in vitro*. In the next phase it will also be of interest to characterize and correlate the types of spinal neurons observed *in vivo* [18] with those observed in this *in vitro* preparation. A GFP construct that specifically expresses in the spinal cord, because it is driven by a neuron specific promoter, was recently reported

[21]. It will be exciting to use that construct in combination with this culture system to gain a better understanding of the molecular mechanisms involved in axon growth and guidance using combined *in vitro* and *in vivo* approaches.

Acknowledgements

Many thanks to A. J. Ashford, P. Scheiffele, G. Tong and T. Wittmann for comments on the manuscript. This work was carried out in the laboratory of Muming Poo. Thanks to Jummer Endres for embryos and for our work setting up Zebrafish in the laboratory at UC San Diego, and thanks to Michelle Page, and Michael Urban for occasional supply of embryos at UC Berkeley. This work was supported by a Human Frontier Science Program long-term fellowship (LT0670/1999) to S. Andersen.

References

1. Harrison RG (1907). Observations on the living developing nerve fiber. *The Anatomical Record* 5: 116–118.

2. Stein E, Tessier-Lavigne M (2001). Hierarchical organization of guidance receptors: silencing of netrin attraction by slit through a Robo/DCC receptor complex. *Science* 291: 1928–1938.
3. Gundersen RW, Barrett JN (1980). Characterization of the turning response of dorsal root neurites toward nerve growth factor. *J Cell Biol* 87: 546–554.
4. Saito M, Wu C-F (1991). Expression of ion channels and mutational effects in giant *Drosophila* neurons differentiated from cell division-arrested embryonic neuroblasts. *J Neurosci* 11: 2135–2150.
5. Yao WD, Rusch J, Poo M, Wu CF (2000). Spontaneous acetylcholine secretion from developing growth cones of *Drosophila* central neurons in culture: effects of cAMP-pathway mutations. *J Neurosci* 20: 2626–2637.
6. Eisen JS, Myers PZ, Westerfield M (1986). Pathway selection by growth cones of identified motoneurons in live zebra fish embryos. *Nature* 320: 269–271.
7. Liu DWC, Westerfield M (1992). Clustering of muscle acetylcholine receptors requires motoneurons in live embryos, but no in cell culture. *J Neurosci* 12: 1859–1866.
8. Westerfield M (1995). *The Zebrafish book* (3rd ed.). Guide for the laboratory use of Zebrafish (*Danio rerio*). University of Oregon Press, Eugene.
9. Eisen JS (1999). Patterning motoneurons in the vertebrate nervous system. *Trends Neurosci* 22: 321–326.
10. Detrich III HW, Westerfield M, Zon LI (1999). In: *Methods in cell biology*, Vol. 59, pp. 3–10 (Detrich III HW, Westerfield M and Zon LI, Eds) Academic press, San Diego.
11. Jontes JD, Buchanan J, Smith SJ (2000). Growth cone and dendrite dynamics in zebrafish embryos: early events in synaptogenesis imaged in vivo. *Nat Neurosci* 3: 231–237.
12. McMahon DG (1994). Modulation of electrical synaptic transmission in Zebrafish retinal horizontal cells. *J Neurosci* 14: 1722–1734.
13. Connaughton VP, Dowling JE (1998). Comparative morphology of distal neurons in larval and adult Zebrafish retinas. *Vision res* 38: 13–18.
14. Spitzer NC, Lamborghini JE (1976). The development of the action potential mechanism of amphibian neurons isolated in culture. *Proc Natl Acad Sci USA* 73: 1641–1645.
15. Tabti N, Alder J, Poo M-M (1998). Culturing spiral neurons and muscle cells from *Xenopus* embryos. In: *Culturing Nerve Cells* (Banker G and Goslin K, Eds) MIT Press, Cambridge.
16. Anderson MJ, Cohen MW (1975). Proceedings: fluorescent staining of acetylcholine receptors in living muscle cells. *J Physiol* 252: 63p–64p.
17. Paw BH, Zon LI (1999). In: *Methods in cell biology*, Vol. 59, pp. 39–43 (Detrich III HW, Westerfield M and Zon LI, Eds) Academic Press, San Diego.
18. Bernhardt RR, Chitnis AB, Lindamer L, Kuwada JY (1990). Identification of spinal neurons in the embryonic and larval Zebrafish. *J Comp Neurol* 302: 603–616.
19. Helmrich A, Barnes D (1999). In: *Methods in cell biology*, Vol. 59, pp. 29–37 (Detrich III HW, Westerfield M and Zon LI, Eds) Academic Press, San Diego.
20. Nasevicius A, Ekker SC (2000). Effective targeted gene ‘knockdown’ in zebrafish. *Nat Genet* 26: 216–220.
21. Higashijima S, Hotta Y, Okamoto H (2000). Visualization of cranial motor neurons in live transgenic zebrafish expressing green fluorescent protein under the control of the *islet-1* promoter/enhancer. *J Neurosci* 20: 206–218.

Address for correspondence: Søren S. L. Andersen, University of California at Berkeley, Department of Molecular and Cellular Biology, 142 Life Sciences Addition #3200, Berkeley, CA 94720-3200, USA
E-mail: soren@sorenandersen1.org