Video Article

Neural Explant Cultures from *Xenopus laevis*

Laura Anne Lowery[1], Anna E.R. Faris[1], Alina Stout[1], David Van Vactor[1]

[1]Department of Cell Biology, Harvard Medical School

Correspondence to: Laura Anne Lowery at Laura_Lowery@hms.harvard.edu, David Van Vactor at Davie_vanvactor@hms.harvard.edu

URL: http://www.jove.com/video/4232
DOI: doi:10.3791/4232

Keywords: Neuroscience, Issue 68, Cellular Biology, Anatomy, Physiology, Growth cone, neural explant, *Xenopus laevis*, live cell imaging, cytoskeletal dynamics, cell culture

Date Published: 10/15/2012

Citation: Lowery, L.A., Faris, A.E., Stout, A., Van Vactor, D. Neural Explant Cultures from *Xenopus laevis*. *J. Vis. Exp.* (68), e4232, doi:10.3791/4232 (2012).

Abstract

The complex process of axon guidance is largely driven by the growth cone, which is the dynamic motile structure at the tip of the growing axon. During axon outgrowth, the growth cone must integrate multiple sources of guidance cue information to modulate its cytoskeleton in order to propel the growth cone forward and accurately navigate to find its specific targets[1]. How this integration occurs at the cytoskeletal level is still emerging, and examination of cytoskeletal protein and effector dynamics within the growth cone can allow the elucidation of these mechanisms. *Xenopus laevis* growth cones are large enough (10-30 microns in diameter) to perform high-resolution live imaging of cytoskeletal dynamics (*e.g.*[2-4]) and are easy to isolate and manipulate in a lab setting compared to other vertebrates. The frog is a classic model system for developmental neurobiology studies, and important early insights into growth cone microtubule dynamics were initially found using this system[5-7] . In this method[8], eggs are collected and fertilized *in vitro*, injected with RNA encoding fluorescently tagged cytoskeletal fusion proteins or other constructs to manipulate gene expression, and then allowed to develop to the neural tube stage. Neural tubes are isolated by dissection and then are cultured, and growth cones on outgrowing neurites are imaged. In this article, we describe how to perform this method, the goal of which is to culture *Xenopus laevis* growth cones for subsequent high-resolution image analysis. While we provide the example of +TIP fusion protein EB1-GFP, this method can be applied to any number of proteins to elucidate their behaviors within the growth cone.

Video Link

The video component of this article can be found at http://www.jove.com/video/4232/

Protocol

Note: We describe the steps in the first two sections only in brief, as excellent protocols with detailed information have been published elsewhere that focus more specifically on these steps (*e.g.*[8-12]). Additionally, the general protocol of working with *Xenopus* spinal neurons in live cell culture has been previously published in a detailed methods article[8]. We highly recommend reviewing that article as a complement to this video, although here we do provide enough information to successfully perform and troubleshoot the neural tube dissection and plating protocol in step 3.

1. *In Vitro* Fertilization of *Xenopus* Eggs

1. Obtain eggs from female frogs that were injected with chorionic gonadotropin (400 units/frog) 12-18 hr before the egg collection. Collect eggs in 1X Marc's Modified Ringer solution (MMR) (0.1 M NaCl, 2.0 mM KCl, 1.0 mM $MgSO_4$, 2.0 mM $CaCl_2$, 5.0 mM HEPES, pH 7.4[9]).
2. Fertilize eggs *in vitro* with minced testes, as described previously[9].
3. After at least 20 min, remove embryo jelly coat by incubating embryos in 2% cysteine in 1X MMR (brought to pH 7.8 with NaOH) for 3-5 min. Wash with 0.1X MMR (or 0.1X MBS (Modified Barth's Saline, 1X recipe: 88 mM NaCl, 1 mM KCl, 0.7 mM $CaCl_2$, 1 mM $MgSO_4$, 2.5 mM $NaHCO_3$, 5 mM HEPES, pH 7.8)) 3-5 times, and keep embryos at room temperature until injections, or if desired, place embryos at 14-18 °C to slow development.

2. Microinjection of RNA

Note: while we utilize RNA injection here, these techniques are not limited to RNA, and DNA, proteins, or modified nucleic acids for gene manipulation can also be used and have been described previously[12].

1. Prior to experiment, RNAs for labeling cytoskeletal or other structures are transcribed from linearized DNA templates using the mMessage mMachine kit (Ambion). mRNAs require a 5' cap and a 3' polyadenylation tail to promote translation and prevent degradation in embryos. pCS2+ is a commonly-used vector for this purpose, and the transcription kit includes a cap analog during the synthesis reaction. We re-suspend transcribed mRNA in nuclease-free ddH_20, at a stock concentration between 500 and 2,000 ng/µl, followed by prompt storage at -80 °C. Prior to injection, dilute RNA in ddH_20 to the appropriate concentration for injection. As only a small volume will be injected into embryos, resuspending in buffer is not necessary. Our final injection concentration is typically 50-200 pg/nl, depending on the construct, and

we usually will inject up to 4 nl per embryo, although many labs traditionally inject up to 10 nl, which is approximately 1% of the total volume of an embryo. RNA can be toxic at high doses, and the dose per embryo generally ranges from 10 pg to 1 ng, although up to 5 ng can be tolerated depending on the particular gene and purity. However, for imaging protein localization, the lowest possible level should be injected to avoid over-expression artifacts. RNA for EB1-GFP in this protocol is used at final amount of 250 pg per embryo.

2. Prepare injection needles by pulling capillary with a needle puller[9], to a tip diameter of ~ 0.2 µm. With a Sutter P-87 puller, we use the following settings: heat 644, pull 125, velocity 70, time 250, but these parameters differ depending on machine and must be re-adjusted after changing the filament (we currently use a 2.5 mm square box filament that is 2.5 mm wide). Under a microscope, break needle tip with forceps at an angle to generate a quill-like shape. There are other methods for breaking and filling injection needles (for example[11,12]), but we fill the needle with RNA by placing a small drop (0.5 - 1 µl) to the back end of the needle. For microinjecting into embryos, there are a number of commercially available injection systems, two of the most common being the Pico-injector (Medical Systems), and the Nanoject (Drummond Scientific) (see[11] for more information). We use the Medical Systems PLI-100 Pico-Injector, which uses compressed gas for a digitally set period of time in order to deliver consistent nanoliter volumes. Injection volume must be calibrated for each new micropipette using a stage micrometer, and injection time should be adjusted to achieve desired amount of injected RNA.
3. Place fertilized embryos at the 1- to 4-cell stage into a plastic dish containing 5% Ficoll in 0.1X MMR. Holding the embryo in place with forceps, or placing in a holding platform (a plastic mesh, with a ~1 mm grid, adhered to the bottom of the dish with plasticine or dental wax), inject desired volume into animal blastomeres (see step 2.1 for details regarding injection volumes). Distribute in several locations throughout embryo, at least one injection into each of the animal blastomeres, to obtain more uniform distribution (for example, for a 4-cell stage embryo, we typically inject 1-2 times in each blastomere, whereas in a 2-cell stage embryo, we inject 2-4 times in each blastomere). A benefit of waiting until the 2-4 cell stage is that you ensure the embryo has begun to cleave normally. However, if time is of the essence, you can start at the 1-cell stage, with little difference in the end result other than more embryos needing to be injected. Older stage embryos can be used as well, and this is particularly useful if specific cell types are targeted for injection, but as we generally prefer more broad expression, we inject in younger embryos to reduce the need for more numerous injections.
4. Transfer injected embryos to a plastic dish containing 0.1X MMR and allow them to develop until stage 20-23[13]. Incubate embryos at 14 to 22 °C depending upon desired speed of development. (For dissecting neural tubes the following day, use ~ 22 °C. For the day after, use ~ 14 °C.)

3. Neural Tube Dissection and Plating

1. Prior to performing the dissections, prepare the culture dishes[14] . First, coat coverslips with enough solution of 200 µg/ml poly-lysine in PBS to pool over the surface of the coverslip, of either Mattek or Lab-Tek culture dishes, and incubate for one hour (alternatively, one could use glass coverslips sitting loose in a Petri dish, for later placement on glass slides for imaging and immunocytochemistry). Aspirate and wash with an excess of PBS 3 times, and let dry. Then coat dishes with 10 µg/ml laminin in PBS (we usually use 500 µl per coverslip, enough to pool over the surface) for one hour at 37 degrees. Aspirate laminin solution and wash 3 times with an excess of PBS, being careful not to let laminin-coated surfaces become exposed to the air interface. Replace PBS with culture media (50% Ringer's, 49% L-15 media, 1% Fetal Bovine Serum, plus 50 µg/ml penicillin/streptomycin and gentamycin, pH 7.4 and filter sterilized). (Ringer's Media, 115 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 10 mM Hepes pH 7.4, 0.5 mM EDTA). While our protocol uses this media, it should be noted that this is an enriched media common for older *Xenopus* retinal ganglion cultures, where neurons have reduced energy reserved. Young spinal cord cultures will survive and grow for more than 24 hr in pure Ringers's Media without additional growth factors, and this is indeed one of the benefits of using this system. Optional: add NT3 and BDNF (to final concentrations of 25 ng/µl each) to culture media to increase axon outgrowth.
2. Because of the variability in the expression of injected mRNA, embryos may exhibit a mosaic of fluorescence. Prior to performing dissections, screen embryos for the presence of fluorescence, to identify those embryos which express the fluorescent fusion protein in the neural tube. Place fluorescent embryos at stage 20-23 into an agarose-coated plastic dish filled with Steinberg's media (58 mM NaCl, 0.67 mM KCl, 0.44 mM $Ca(NO_3)_2$, 1.3 mM $MgSO_4$, 4.6 mM Tris, pH to 7.8 then autoclaved)[5]. To make the agarose-coated dish, coat bottom of plastic dish with melted 1% agarose in 0.1X MMR and let harden. This provides a soft surface so that damage does not occur to the fine forceps and tungsten needles used during the subsequent dissection steps. Dissection of embryos beyond stage 23 is possible, but it is more challenging as tissues adhere more closely to each other and thus require longer treatment with collagenase.
3. Under a dissecting scope, remove the vitelline membrane with fine forceps, and then isolate the entire dorsal portion of the embryo, by making a series of incisions. While one forceps hold the embryo in place, use the second to make an incision on the side of the embryo to expose the hollow interior. Then, use both forceps to pinch along the tissue between the dorsal and ventral halves of the embryo, thereby cutting the embryo in half to isolate the dorsal portion containing the neural tube.
4. Place the dorsal explant in an Eppendorf tube with 2 mg/ml collagenase in Steinberg's media for 15-20 min on a rotator, to loosen tissues, and then pipette dorsal explant into a plastic agarose-coated dish with fresh Steinberg's solution. Alternatively, the explant can be placed in a Petri dish containing the collagenase solution for 15 min, where the entire dissection could then be carried out.
5. Using a pair of forceps, gently dissect the neural tube from the dorsal epidermis and the ventral notochord. Slide the tip of the forceps between the epidermis and underlying tissue and slowly pull back the epidermis, revealing the neural tube beneath. Then, use one forceps to hold the tissue and another to slide the tip between the neural tube and notochord. Finally, use forceps to remove the somites on either side of the tube.
6. Transfer the neural tube to an agarose-coated dish filled with culture media, as described in step 3.1.
7. After collection of several neural tubes, cut them into numerous pieces (approximately 20, if the whole tube is isolated) using sharpened tungsten needles or forceps, and then plate the pieces in culture dishes filled with medium (prepared in step 3.1), spreading out the explants evenly in rows.
8. After plating, do not move the dishes because this would disturb the attaching cells. Incubate the plated neural tube explants around 20-22 °C. We typically leave the dish of cells on the bench at room temperature overnight. One of the benefits of *Xenopus laevis* neurons is that a special incubator for cell culture that regulates CO_2 levels or temperature is not needed. Neurites and growth cones can be observed and imaged at room temperature 12-24 hr after plating, although outgrowth can be accelerated with the addition of growth factors. In general, expression of a RNA or plasmid product will result in variable expression between cells, and thus there may be a range of fluorescence expression levels between growth cones. We have observed RNA expression of fluorescent proteins to persist beyond 48 hr after plating, although this depends on the particular construct.

4. Representative Results

After following the protocol, which is summarized in **Figure 1A**, healthy, correctly-dissected and cultured neural explants will send out numerous axons or neurites in every direction on laminin/poly-lysine substrate, shown in **Figure 1B**. The growth cones at the tips of the axons can be imaged by either DIC optics, as seen in **Figure 1C**, to image overall motility dynamics, or high-resolution fluorescence microscopy to examine the localization of fluorescently-tagged cytoskeletal proteins, for example, EB1-GFP is shown in **Figure 1D**.

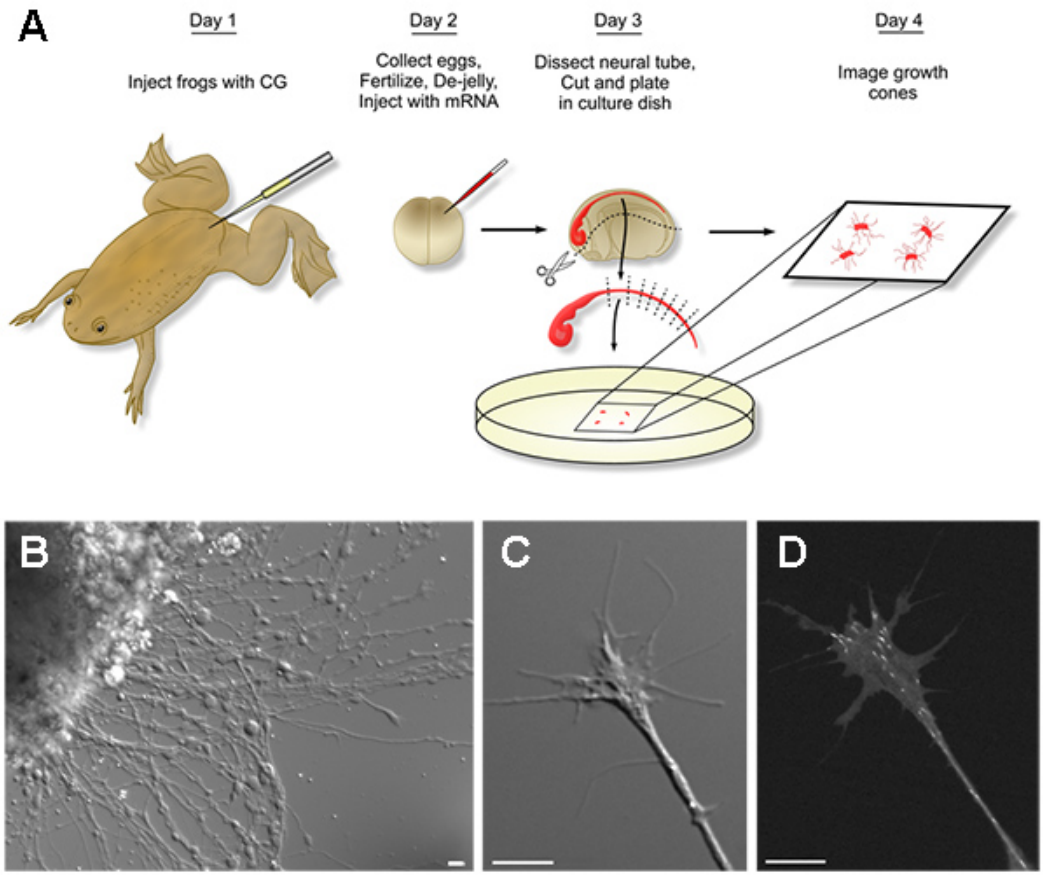

Figure 1. Summary of experimental protocol and expected outcome. A) Protocol flow-chart. See movie for details regarding dissection on day 3. The entire neural tube should be cut into about 20 equally-sized pieces and spread out evenly in rows on the coverslip. In this cartoon, scissors are depicted to represent cutting the tissue with fine forceps. CG chorionic gonadotropin B) DIC image of axons or neurites growing out of explant (explant at top left corner). C) Higher magnification image of growth cone at tip of a growing axon. D) Fluorescence microscopy image of EB1-GFP in growth cone. Scale bar 10 µm. Click here to view larger figure.

Discussion

Xenopus laevis neural explants send out neurites in a very robust manner by 24 hr after plating on the laminin/poly-lysine substrate if conditions are appropriate. With this substrate, growth cones are highly motile and can achieve axon lengths of up to 1 mm, extending in all directions outward from the explant, although typical lengths are 100 µm or more. If neurites do not grow out, there are a limited number of reasons for this to be the case. One possibility is that the neural explants did not properly adhere to the dish. Make sure to not disrupt attachments by accidentally moving the dish after plating. Also, ensure that laminin- and poly-lysine-coated dishes were made correctly with all freshly-prepared reagents. Another possibility for lack of outgrowth is that cell culture media conditions may not be optimal. Double check protocol and calculations to ensure that all media solutions have been correctly formulated. Finally, it is possible that embryos from which the explants were obtained might be unhealthy, although if embryos can develop to the neural tube stage, this is less likely to be the issue. However, it is best to keep some whole intact embryos cultured in 0.1X MMR along with dissections to ensure that continued development occurs.

The most critical step of this protocol is the neural tube dissection. Compared to many model system embryo micro-dissections, this is a relatively simple technique and *Xenopus laevis* embryos are one of the largest and most forgiving of organisms for dissecting. However, if the experimenter is completely new to performing embryonic dissections, this step may take several hours to perfect (best spread out over 2-3 sessions). After the collagenase treatment, some prefer to next remove the mesoderm, notochord, and somites, followed by the epidermis, thus resulting in the isolated neural tube, while others prefer to start with epidermis removal and then separating the other tissues from the neural tube. It is recommended to try both methods to determine which works best for each researcher. Also, be careful to not put too much strain on the neural tube during its isolation. It is better to remove the other tissues from the neural tube, rather than remove the neural tube from the other

tissues, in order to reduce tension on the tube and prevent it from fragmenting prematurely. Finally, if surrounding tissues are too adherent to the neural tube, then place the explant back into the collagenase solution for several more minutes. This will make the separation of the neural tube from the surrounding tissues, especially the epidermis, much easier. The length of collagenase treatment depends on the stage of the embryo, as older embryos require longer treatments, up to 45 min or more for stage 28 embryos.

In addition to culturing neural tube explants, neural tubes could also be dissociated prior to plating, in order to image single cells. Protocols for this method are described in [8, 15]. A benefit of this method is that neuronal morphology and neurite length can be examined, as the cell body is not obscured inside the explant. Moreover, neural explants can also be cultured on various patterned substrates. For example, the guidance cue "stripe assay" has been used to assess changes in growth cone dynamics in response to encountering substrate borders in culture [16, 17]. A detailed protocol for preparing striped patterns of cues has been described [18]. This can allow for the analysis of cytoskeletal dynamics while the growth cone undergoes guidance cue steering decisions.

There are a number of benefits to using *Xenopus laevis* for neural explant culturing experiments. Compared to other systems, culturing these primary neurons are relatively easy to obtain and require low cost. As they can be cultured and imaged at room temperature, expensive incubators are not required. *Xenopus* embryos are easily accessible and can be obtained in large quantities, they develop rapidly, and they are large enough to dissect with minimal training. Thus, this system is particularly suited for teaching laboratories.

Another advantage of using frog embryos is that they are amenable to manipulations of gene, mRNA, or protein expression levels, depending on the needs of a particular experiment. For example, a benefit of using mRNA is that the concentration and injection volume can be carefully titrated to control the resulting expression level, while injecting DNA, on the other hand, allows for the creation of transgenic embryos in which gene expression can be controlled by spatial or temporal specific promoter (*e.g.* [19]). Thus, this neural explant method can be applied to multiple situations for understanding the behavior and function of proteins-of-interest during axon outgrowth and growth cone dynamics.

Disclosures

No conflicts of interest declared.

Acknowledgements

The authors would like to thank Bob Freeman for training and the Kirschner lab for the use of the frog facility, and members of the Van Vactor lab for support. We thank the Nikon Imaging Center at Harvard Medical School for assistance with light microscopy for the images in **Figure 1**. This work was funded by the following: NRSA NIH fellowship and NIH K99 fellowship to L.A.L, Basic Science Partnership funding (https://bsp.med.harvard.edu/) to AEF, and NIH RO1 NS035909 to D.V.V.

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