Ex vivo oculomotor slice culture from embryonic GFP-expressing mice for time-lapse imaging of oculomotor nerve outgrowth

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LONG ABSTRACT:

Accurate eye movements are crucial for vision, but the development of the ocular motor system, especially the molecular pathways controlling axon guidance, has not been fully elucidated. This is partly due to technical limitations of traditional axon guidance assays. To identify additional axon guidance cues influencing the oculomotor nerve, an *ex vivo* slice assay to image the oculomotor nerve in real-time as it grows towards the eye was developed. E10.5 *Isl^{MN}-GFP* embryos are used to generate *ex vivo* slices by embedding them in agarose, slicing on a vibratome, then growing them in a microscope stage-top incubator with time-lapse photomicroscopy for 24–72 hours. Control slices recapitulate the *in vivo* timing of outgrowth of axons from the nucleus to the orbit. Small molecule inhibitors or recombinant proteins can be added to the culture media to assess the role of different axon guidance pathways. This method has the advantages of maintaining more of the local microenvironment through which axons traverse, not axotomizing the growing axons, and assessing the axons at multiple points along their trajectory. It can also identify effects on specific subsets of axons. For example, inhibition of CXCR4 causes axons still within the midbrain to grow dorsally rather than ventrally, but axons that have already exited ventrally are not affected.

SHORT ABSTRACT:

An *ex vivo* slice assay allows oculomotor nerve outgrowth to be imaged in real time. Slices are generated by embedding E10.5 *Isl^{MN}:GFP* embryos in agarose, slicing on a vibratome, and growing in a stage-top incubator. The role of axon guidance pathways is assessed by adding inhibitors to the culture media.

DISCLOSURES:

The authors have nothing to disclose.

Keywords

oculomotor; congenital cranial dysinnervation disorder; axon guidance; eye movements; time-lapse imaging; slice culture

INTRODUCTION:

The ocular motor system provides an elegant system for investigating axon guidance mechanisms. It is relatively uncomplicated, consisting of three cranial nerves innervating six extraocular muscles (EOMs) which move the eye, and the levator palpebrae superioris (LPS) which lifts the eyelid. The oculomotor nerve innervates the LPS and four EOMs - the inferior oblique and the medial, inferior, and superior rectus muscles. The other two nerves, the trochlear and abducens, each only innervate one muscle, the superior oblique and lateral rectus muscle, respectively. Eye movements provide an easy readout, showing if innervation was appropriate, missing, or aberrant. Additionally, there are human eye movement disorders that result from deficits in neuronal development or axon guidance, collectively termed the congenital cranial dysinnervation disorders (CCDDs)¹.

Despite these advantages, the ocular motor system is rarely used in axon guidance studies^{2–10}, due to technical drawbacks. *In vitro* axon guidance assays have many disadvantages¹¹. Co-culture assays, in which neuronal explants are cultured together with explants of target tissue¹² or transfected cells¹³, depend on both symmetry of the explant and precise positioning between the explant and target tissue. Stripe assays^{14,15}, in which two cues are laid down in alternating stripes and axons are assessed for preferential growth on one stripe, only indicate that one substrate is preferable to the other, not that either is attractive or repulsive, or physiologically relevant. Microfluidics chambers can form precise chemical gradients, but subject growing axons to shear stress^{16–18}, which can affect their growth. Moreover, in each of these approaches, collecting explants or dissociated cells requires that outgrowing axons be axotomized and thus these assays actually examine axon regeneration, rather than initial axon outgrowth. Finally, these *in vitro* approaches remove the microenvironment that influences axons and their responses to cues along different points of their course, and traditionally only test one cue in isolation. Compounding these disadvantages, the small size of each nucleus in the ocular motor system makes dissection technically challenging for either explants or dissociated cultures. Additionally, primary cultures of ocular motor neurons are usually heterogeneous, have significant cell death, and are density dependent, requiring pooling of cells from multiple embryos (Ryosuki Fujiki, personal communication). *In vivo* methods, however, including knockout mouse models, are inappropriate to use for screening, given the time and expense required.

Methods developed to culture whole embryos¹⁹ allow labeling of migrating cells²⁰ or blockade of specific molecules²¹, but whole embryo cultures require incubation in roller bottles which precludes real-time imaging of labeled structures. Surgical techniques that allow manipulation of the embryo and then subsequent further development either in the uterus or in the abdomen of the mother (maintaining the placental connection)²² are also possible, but these also do not allow time-lapse imaging.

To overcome the obstacles of in vitro assays and allow rapid screening of signaling pathways, an *ex vivo* embryonic slice culture technique was developed²³, adapted from a previously published protocol for peripheral nerve outgrowth²⁴. Using this protocol, the developing oculomotor nerve can be imaged over time in the presence of many of the surrounding structures along its trajectory, including EOM targets. By adding small molecule inhibitors, growth factors, or guidance cues to the culture media, we can assess guidance perturbations at multiple points along the axon trajectory, allowing more rapid assessment of potential growth and guidance factors.

PROTOCOL:

All animal work described here was approved and performed in compliance with the Boston Children's Hospital Institutional Animal Care and Use Committee (IACUC) protocols.

1. Timed matings
 - 1.1. Place *ISL^{MN}:GFP* (Islet Motor Neuron Green Fluorescent Protein; MGI: J:132726; Jax Tg(Isl-EGFP*)1Slp/J Stock No: 017952) male and female mice together overnight. Weigh females and record weights prior to mating.

Note: *ISL^{MN}:GFP* specifically labels motor neurons with a farnesylated GFP that is not cytotoxic, localizes to the cell membrane of motor neurons and their axons, and allows the nerves to be visualized during development²⁵. Other fluorescently labeled lines could also be used.
 - 1.2. Check for vaginal plugs in early morning. The date a plug is identified is designated as E0.5.
 - 1.3. Confirm pregnancy using weight gain and/or ultrasound at E10.5. Pregnant dams should have gained at least one gram. Embryos can be seen on ultrasound at E10.5.
2. Prepare reagents and vibratome for slice culture
 - 2.1. Prepare 500 mL of slicing buffer: add 5 mL HEPES and 5 mL penicillin/streptomycin to 500 mL Hank's Balanced Salt Solution (HBSS) without Ca²⁺ and Mg²⁺. Chill to 4 °C. **Note:** Extra slicing buffer should be stored at 4 °C and can be used for future slice culture experiments.
 - 2.2. Prepare 50 mL of culture media: In a sterile hood, add 12.5 mL HBSS, 12.5 mL Fetal Bovine Serum, 250 µL glucose, 250 µL L-glutamine, 125 µL HEPES to 24.4 mL Fluorobright DMEM. (Final concentrations: Fluorobright DMEM with 25% HBSS, 25% FBS, 0.5% glucose, 1 mM glutamine, and 2.5 mM HEPES.). Warm culture media to 37 °C in a sterile water bath. **Note:** Culture media can be stored at 4 °C for up to 3 weeks.

- 2.2.1.** In a sterile hood, add 1.5 mL culture media to each well of a 6-well plate. Add a Millicell cell culture insert to each well. Place in a sterile 37 °C 5% CO₂ incubator.
- 2.3.** Prepare 4% low-melting temperature agarose: dissolve 2 g low-melting temperature agarose in 50 mL sterile PBS. Microwave in 30–60 second intervals until fully dissolved. Place in 40 °C water bath to keep liquid. **Note:** Extra agarose can be stored at RT and melted for future slice culture experiments.
- 2.4.** Set up vibratome: Place new blade on vibratome. Check settings: thickness 400–450 µm. Pre-chill vibratome stage. Place ice in outer chamber. **Note:** Use a chamber and stage dedicated to live slices. Do not use the same vibratome chamber for fixed tissue as residual fixative could be damaging to slices.
- 2.5.** Prepare microscope stage top incubator to 37 °C and 5% CO₂.
- 2.6.** Prepare for dissection: Clean surgical instruments and spray with 70% ethanol. Fill two petri dishes with HBSS, place on ice. Open a 12 well tissue culture plate and place the lid on ice with the underside facing up. Open a 6 well tissue culture plate and place cell culture membrane inserts and 1.5 mL cell culture media in each well. Pre-warm the plate in a 37 °C and 5% CO₂ incubator.
- 3.** Harvest E10.5 embryos and prepare slices.
- Note:** All steps from this point should be done as quickly as possible. Keep embryos on ice at all times.
- 3.1.** Euthanize the pregnant dam (E10.5) in a CO₂ chamber. Perform cervical dislocation.
- 3.2.** Spray the abdomen with 70% ethanol. Cut open the abdomen with scissors and remove the uterus and place it in a petri dish with ice cold HBSS to quickly wash away blood. Move the washed uterus to a second ice cold HBSS filled petri dish.
- 3.3.** Under a dissecting scope, remove the embryos from the uterine horn and individual amniotic sacs. Place embryos on the underside of the lid of a 12 well plate. Keep on ice.
- 3.4.** Under a dissecting scope, use filter paper to remove any liquid surrounding each embryo. **Note:** Embryos will stick to the filter paper if touched.
- 3.5.** Embed embryos in agarose.
- 3.5.1.1.** Pour melted agarose over each embryo to cover it. Keep on ice.

3.5.1.2. As soon as the agarose has hardened, flip each embryo and pour additional agarose on the other side. Keep on ice.

- 3.6.** Using a fluorescent dissecting scope, trim the agarose around each embryo so it will be oriented properly when glued to the vibratome stage. The oculomotor nucleus and early axon outgrowth are fluorescent. Align the embryo so that the nucleus, outgrowing axons, and eye form a line, and trim the agarose with a razor blade cut parallel to this line (dorsal to the embryo, see Figure 1A). This will be the side glued to the vibratome stage (the embryo will be positioned on its back, head closest to the vibratome blade). A line between the oculomotor nucleus and eye should be parallel to the vibratome blade.
- 3.7.** Fill the vibratome chamber with ice-cold slice buffer. Superglue the embryo to the vibratome stage so that the blade will be parallel with the oculomotor nucleus and eyes. Once the superglue is dry, submerge the vibratome stage so embryo is oriented facing away from blade.
- 3.8.** Slice 400–450 μm slices. Collect each slice with a sterile transfer pipet. Place into cold slicing buffer.
- 3.9.** Under the dissecting scope, choose the slice containing the oculomotor nuclei and eyes. Using a sterile transfer pipet, place it on the cell culture insert in the 6 well plate. Return the plate to the 37 °C incubator. Alternatively, have one person slicing and another placing the slices. Minimize the time between slicing and placing into incubator.
- Note:** Slices should be oriented in a way that the maximum fluorescence emitted from the nuclei and axons is closest to the imaging microscope objective. On an inverted microscope, the slices should be placed on the membrane with the nuclei and axons closest to the objective underneath the plate.
- 3.10.** Remove the residual agarose from the vibratome stage, superglue the next embryo to the stage and repeat steps 3.7–3.9 until all embryos have been sliced and plated.
- 3.11.** Add inhibitor or recombinant molecule of choice to media in each well to create a dose-response curve. Dilute in appropriate solvent. Note: If using DMSO, use only tissue culture grade DMSO. Alternatively, protein-eluting beads can be placed in specific locations on the slice.
- 3.12.** Place on microscope in 37 °C 5% CO₂ chamber. Set microscope to take phase contrast and fluorescent photographs of each slice every 30 minutes (or more often if desired). Slices can be maintained for 48–72 hours.

REPRESENTATIVE RESULTS:

Normal Results:

Figure 1 provides a schematic of the experiment. Starting as early as E9.5 in mouse, the first axons begin to emerge from the oculomotor nucleus²⁶. By E10.5, a fasciculated oculomotor nerve, which contains the early pioneer neurons, can be seen in the mesenchyme. There is significant variability between embryos at E10.5 (even within the same litter) in how far the nerve has progressed towards the orbit, likely due to developmental differences of a few hours. During normal *in utero* development, the first GFP-positive oculomotor axons reach the orbit/eye over the next 18–24 hours (by E11.5), and then begin branching to their final targets²⁷. In slice culture, this timing is recapitulated (Figure 2, Video 1). The slice grows and expands (in all directions) for up to 72 hours, and axons can be seen extending from the nucleus towards the eye. We have found the first 36–48 hours most useful for assessing oculomotor axon outgrowth. Motor neurons can sometimes be seen moving within the nucleus (not shown). In some slices, depending on orientation, the trochlear nucleus is also present. Trochlear axons extend laterally within the slice and often stall, because their normal course is to exit the trochlear nucleus laterally, before turning dorsally and caudally, which would be out of the slice. Occasionally, the trochlear axons seem to join with the oculomotor axons and turn towards the eye, which makes the slice difficult to interpret, as those axons may have a secondary effect on the guidance of the oculomotor axons.

Example of inhibiting an important axon guidance pathway: Inhibiting CXCR4 signaling causes dorsal rather than ventral growth of oculomotor axons.

We assessed the role of CXCR4 signaling on oculomotor development by adding 1 µg/ml (1.26 µM) of AMD3100, a water-soluble small molecule inhibitor of CXCR4²⁸, directly to the culture media as soon as the slice culture is prepared. The oculomotor axons can then be seen exiting the oculomotor nucleus dorsally and growing away from the orbit (“backwards”) (Figure 2, Video 2). Those axons that had already left the midbrain and were en route to the orbit at E10.5 continue on their path. Inhibition of CXCR4 also affects overall growth of the slice.

Orientation of the slice is crucial.

Slices are not interpretable if they are not oriented properly. Some examples of improperly oriented slices are shown in Figure 3. If the embryo is tilted to its side, only one oculomotor nucleus is in the slice (Figure 3A). If the embedded embryo is tilted too far towards its back, the eyes are not in the slice, and instead the upper limb and hindbrain or spinal cord may be in the slice (Figure 3B). In this case, the normal trajectory of the oculomotor axons is not present, and they tend to grow randomly. During placement of the slice on the culture membrane, it can become folded (Figure 3C).

Solvents can be toxic.

Care should be taken when dissolving inhibitors or growth factors in organic solvents. Figure 4 shows an example of a slice that died after the addition of ethanol to the media. 118 µl was added to 1.5 mL of media (final concentration 7.8%), and this high concentration

proved toxic. To prevent this, dissolve solutes in the minimum amount of solvent possible (to the limit of solubility). Whenever possible, dissolve in water. If DMSO is used, ensure that it is sterile, tissue culture grade DMSO.

DISCUSSION:

This *ex vivo* slice culture protocol provides significant advantages over traditional axon guidance assays²³. The size of each cranial motor nucleus is not a limiting factor, and no difficult dissection is necessary. The endogenous microenvironment through which the axons travel is maintained, allowing modification of one signaling pathway while maintaining other signaling pathways. Additionally, effects can be assessed at different points along the axon trajectory. Since axon guidance requires multiple cues, and combinations of cues, along the path²⁹, this provides a significant advantage. Unlike in dissociated or explant cultures, growing axons are not cut in this preparation, so initial axon outgrowth can be assessed, rather than axon regeneration. In most other guidance assays, axon regeneration is actually being assessed, rather than initial axon outgrowth. Avoiding axotomy also avoids significant potential confounding factors because the neurons are not damaged or under stress.

The two most critical steps are the orientation of the embryos for slicing and minimizing the time between euthanasia of the mother and placement of the slices in the incubator. Embryos must be kept on ice at all times. For orientation, we have tried several different molds, but because of variability between embryos at this age, we have found that orientation by hand, based on fluorescence under the dissecting microscope, is most effective. Orientation by hand limits the embryonic time window that can be studied, however. Earlier than E10.0, orienting the slice properly is challenging because the eye is difficult to see and very few CN3 axons can be seen. This does mean, however, that we cannot use this method to study the earliest pioneer axon outgrowth, as we need to allow those axons to grow out part way to be able to orient the slice.

There are several limitations to the oculomotor slice culture preparation. It is most useful for studying early and intermediate guidance decisions, rather than terminal branching decisions. At E10.5, the EOMs are present only as a muscle anlage. Even at later time points, with the current imaging technology, individual target EOMs cannot be distinguished within the slice. Specific terminal branching decisions of the oculomotor axons therefore cannot be assessed with the current conditions. Because the slices are grown on porous tissue culture membranes, post-culture separation from the membrane is difficult and often causes tissue damage, making antibody staining and remounting for additional imaging unsuccessful. Although the axons are not cut, thereby minimizing neuronal damage, the surrounding tissues are cut and therefore damaged, which could affect signaling cue expression or release. The ideal time window is also limited. As noted above, earlier than E10.0 orientation of the slice is difficult, and after E11.5, many axons have already reached the orbit, so intermediate guidance decisions have already been made.

The preparation requires a microscope with stage-top incubator, automatic stage, and time-lapse capability. The objectives and camera need to be optimized for imaging of thick

specimens with no clearing. Resolution at the level of a single growth cone, as is possible with some culture preparations, is not possible with the current imaging set-up. The imaging parameters could, however, potentially be adapted to allow higher resolution, with confocal or 2-photon microscopy, focusing on a small area of each slice (potentially adjusting as the slice as axons grow). Using the current epifluorescent microscope and imaging every 30 minutes, we have not seen photobleaching; confocal microscopy and more frequent imaging could be used, but photobleaching would become a potential problem.

Because small molecule inhibitors may have off-target effects, including on overall growth and health of the slice, this assay is most useful as a screening tool. Results need to be verified by other methods, such as knockout mouse models. For example, with CXCR4 inhibition, there is mild decreased overall growth of the slice (see Figure 2), consistent with the multiple functions of CXCR4. In mouse models, however, the axon phenotype seen in the slice was recapitulated²³.

This protocol could be modified to study other cranial nerve populations, although for each the proper orientation of the slices and timing would have to be empirically determined. Additionally, mice with different fluorescent labels could be used that mark different subsets of neurons and/or that turn on to at earlier or later embryonic ages.

The slice culture can be manipulated in multiple ways. We show here an example of blocking receptor signaling with a small molecule inhibitor. Alternatively, antibodies (either receptor blocking or receptor activating) or recombinant proteins, such as axon guidance cues or growth factors, could be added to the media. To evaluate for directional effects on axon guidance, ligand-secreting beads can be placed on the slice in specific locations. Using any of these methods, many other axon guidance pathways can be identified and studied in the ocular motor system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: Schematic.

E10.5 *Isl^{MN}-GFP* embryos (**A: photo, B: schematic**) are sliced on a vibratome (400–450 μm thick) so that sections include both the oculomotor nucleus and the orbit. Parallel dashed lines in A and B denote the location of the vibratome cuts. The lower dashed line in A shows where the agarose should be trimmed and glued to the vibratome stage. **C:** Sections are laid flat on a tissue-culture membrane, allowing exchange of nutrients and gasses, and placed in a stage-top incubator for time-lapse microscopy. At this stage, inhibitors can be added to the growth media. **D:** Cultures can be maintained for 24–72 hours, and the oculomotor axons can be seen growing to the eye. Adapted with permission from Whitman, et. al. 2018²³.

Figure 2: Inhibition of CXCR4 causes CN3 misrouting.

Slice cultures from E10.5 Isl-GFP embryos imaged at 0, 12, 24, and 36 hours in culture. The top panel shows a wild-type control embryo and CN3 (green) grows toward the eyes and branches extensively over the course of 36 hours. Also see Video 1. The bottom panel shows a slice with 1 µg/ml AMD3100 (specific inhibitor for CXCR4) added and axons exit the oculomotor nucleus dorsally. Those that had already exited ventrally continue towards the eye. Also see Video 2. D: dorsal, V: ventral, E: eye, N: oculomotor nucleus, SMN: spinal motor neurons, arrows: oculomotor axons (white: wildtype projection, yellow: aberrant projections). Scale bar equals 200 µm. Also see Videos 1 and 2. A similar figure showing other examples was presented in Whitman, et. al. 2018²³.

Figure 3: Orientation of the slices is crucial.

Initial images (time 0 hours) of representative misoriented slices. **A** shows a slice in which the embryo was tilted to the side such that projecting CN3 axons were axotomized.

Projecting axons are only present on the right side of the slice (arrow). **B** shows a slice in which the embryo was tilted back so CN3 nuclei with projecting axons (arrow) are visible bilaterally, but the eyes are not present in the slice. Instead spinal motor neurons (SMN) and motor neuron projections to the limbs can be seen. **C** shows a slice that folded during placement on the membrane. Slices such as those shown here should be discarded. Scale bars represent 500 μm in each panel.

Figure 4: Solvents can be toxic.

Time-lapse imaging of slice cultures from E10.5 Isl-GFP embryos at 0 and 12 hours in culture. A slice cultured in media with no additives (**A-B**) grows normally for 12 hours with CN3 axons projecting towards the eye. A slice cultured in media with a high concentration of ethanol (7.8%) (**C-D**) does not grow normally. At 12 hours, CN3 has not grown and is barely visible, and the tissue has started to disintegrate. Scale bars represent 500 μm in each panel.