Protocol

Live Imaging of Cytoskeletal Dynamics in Embryonic *Xenopus laevis* Growth Cones and Neural Crest Cells

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The cytoskeleton is a dynamic, fundamental network that not only provides mechanical strength to maintain a cell's shape but also controls critical events like cell division, polarity, and movement. Thus, how the cytoskeleton is organized and dynamically regulated is critical to our understanding of countless processes. Live imaging of fluorophore-tagged cytoskeletal proteins allows us to monitor the dynamic nature of cytoskeleton components in embryonic cells. Here, we describe a protocol to monitor and analyze cytoskeletal dynamics in primary embryonic neuronal growth cones and neural crest cells obtained from *Xenopus laevis* embryos.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Ficoll (5% in 0.1× MMR)
Marc's modified Ringer's (MMR) (10×, pH 7.4) <R>
mRNA encoding fluorophore-tagged protein of interest (see Step 12 and Table 1)

> *Synthesize capped mRNAs in vitro using the mMessage mMachine kit (Invitrogen). Determine the concentration of mRNAs using a Nanodrop. Verify the presence and integrity of the mRNA on an RNA gel.*

PBS (1×; sterile)

> *Dilute 10× PBS (pH 7.4, RNase-free; Invitrogen AM9625) in deionized H_2O to make 1× PBS.*

Reagents for neural crest cell culture only (see Steps 5–10 and Steps 24–28)
    Fibronectin (20 µg/mL in 1× PBS; Sigma-Aldrich F1141)
    Gelatin (Sigma-Aldrich G1890)
    Plating culture medium for neural crest cells <R>
Reagents for neural tube culture only (see Steps 1–4 and Steps 16–23)
    Crystalline collagenase (2 mg/mL in Steinberg's medium)
    Laminin (10 µg/mL in 1× PBS; Sigma-Aldrich L2020)

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TABLE 1. Useful cytoskeletal markers to visualize microtubules (MTs), actin filaments, or focal adhesions

Marker	Labels	Purpose	Concentration range per embryo	Imaging technique	Analysis software
EB1-3 (Stepanova et al. 2003)	MT plus-end	MT growth dynamics	100 pg–300 pg mRNA	Spinning disk confocal microscopy (SDCM)	plusTipTracker (Applegate et al. 2011; Stout et al. 2014)
MACF-43 (Honnappa et al. 2009)	MT plus-end	MT growth dynamics	100 pg–300 pg mRNA	SDCM	plusTipTracker (Stout et al. 2014; Applegate et al. 2011)
Fluorophore-tagged tubulin	MTs	Uniform labeling to visualize MTs	900 pg mRNA	SDCM	Fiji (ImageJ) (Schindelin et al. 2012)
		Speckle labeling to assess MT flow rates	300 pg mRNA	Total internal reflection microscopy (TIRF)	QFSM software (Danuser and Waterman-Storer 2006; Mendoza et al. 2012)
Actin monomer (G-actin)	Actin	Speckle labeling to assess actin dynamics	300 pg mRNA	TIRF	QFSM software (Danuser and Waterman-Storer 2006; Mendoza et al. 2012)
LifeAct -GFP (Riedl et al. 2008), Utrophin (Burkel et al. 2007), F-Tractin (Schell et al. 2001)	F-actin	Actin dynamics	LifeAct/utrophin may affect dynamics dependent on the concentration. Optimization may be required. 60–300 pg can be used to begin.	SDCM, TIRF	Fiji (ImageJ) (Schindelin et al. 2012)
Paxillin (Robles and Gomez 2006), PAK2 (Santiago-Medina et al. 2013), focal adhesion kinase (Myers and Gomez 2011), zyxin, vinculin, talin	Focal adhesion	Focal adhesion dynamics	50–75 pg DNA, 250–500 pg mRNA	TIRF	FAAS (Berginski and Gomez 2013)

Plating culture medium for neural tube explants <R>

This is an enriched medium that is commonly used for older Xenopus *retinal ganglion cultures, where neurons have reduced energy reserves. Young spinal cord cultures will survive and grow for >24 h in pure Ringer's solution <R> without additional growth factors; this is one of the benefits of using this system.*

Poly-L-lysine (PLL; 100 µg/mL; Sigma-Aldrich P4832)

PLL is reconstituted in sterile H_2O and diluted to working concentration (100 µg/mL) with 1× PBS.

Steinberg's medium <R>

Xenopus laevis embryos

These must be staged appropriately for neural crest (stage 18) or neural tube (stage 20–23) isolation (see Step 15; Nieuwkoop and Faber 1994; Wlizla et al. 2018). Injections are often made at the two- to four-cell stage (see Step 11).

Equipment

Equipment for neural crest cell dissections only (see Steps 24–28):

Eyelash knife (or insect pins; see Step 26)

Plasticine clay-coated Petri dish <R>

Equipment for neural tube dissections only (see Steps 16–23):

Agarose-coated Petri dish <R>

Alternatively, use a Sylgard 184-coated Petri dish <R>. Agarose- and Sylgard-coated plates can be used multiple times.

Electrolytically sharpened tungsten needles

Fine forceps (Dumont #5 or equivalent)

Fluorescence dissecting microscope

Glass injection needles

> *See Lowery et al. (2012) for instructions on how to make injection needles.*

Image analysis software (see Step 30 and Table 1)

Imaging setup (see Step 29)

> *Use a spinning disk confocal microscope (SDCM), total internal reflection fluorescence (TIRF) microscope, or widefield epifluorescence scope equipped with an appropriate objective. Imaging cytoskeletal dynamics will generally require a relatively high-magnification objective (60× or 100×). Our SDCM rig uses a 63× Plan Apo 1.4 NA objective, and our TIRF setup uses a 60×, 1.49 NA objective. For detection, a growing number of modern CCD and scMOS cameras will be suitable; ensure that the chip size, sensitivity, and camera speed are appropriate. Our SDCM uses an Orca Flash 4 CCD; our TIRF imaging is typically acquired with an Andor ELYRA scMOS.*

Incubator(s) set at the appropriate temperature (see Steps 1, 3, 15, 23, and 28)

MatTek glass bottom (No. 1.5) culture dish (35-mm)

Petri dish (60-mm × 15-mm) and plastic mesh with 1-mm grid to fit in Petri dish (see Step 11)

Rotator at room temperature

METHOD

Culture Dish Coating

> *Coat imaging chambers with the appropriate coating reagents depending on the type of culture. For neural tube cultures, begin with Step 1; for neural crest cell cultures, begin with Step 5.*

Neural Tube Culture Plates

1. Add 500 µL of 100 µg/mL poly-L-lysine (PLL) into the center of a 35-mm MatTek glass bottom (No. 1.5) culture dish and incubate for 1 h at 37°C.

2. Rinse off PLL by washing the dish with 1× sterile PBS three times.

3. Add 500 µL of 10 µg/mL laminin into the center of the PLL-coated culture dish and incubate for 1 h at 37°C.

4. Rinse off laminin by washing the dish with sterile 1× PBS three times and use immediately for neural tube culture (see Steps 16–23).

Neural Crest Cell Culture Plate

5. Heat 2 mg/mL gelatin dissolved in distilled water in the microwave until boiling; allow it to boil 5–10 sec until dissolved, then allow to cool for 5 min.

6. Add 500 µL of cooled gelatin into the center of a 35-mm MatTek glass bottom (No. 1.5) culture dish.

7. Rotate gently for 25 min at room temperature.

8. Discard the gelatin and rinse the dish with 1× sterile PBS several times.

9. Add 500 µL of 20 µg/mL fibronectin and incubate dish overnight at 4°C.

10. On the next day remove the fibronectin. Rinse the plate with sterile 1× PBS several times and use to culture cells (see Steps 24–28).

Injection of RNA Encoding Fluorescently Tagged Cytoskeleton Proteins

> *More information regarding embryo injections, including injection needle preparation, can be found in Lowery et al. (2012).*

11. Place a plastic mesh with a 1-mm grid on the bottom of a Petri dish (60-mm × 15-mm) and fill the dish with 5% Ficoll in 0.1× MMR. Position *Xenopus* embryos on the mesh grid to hold them in place during injections.

    Injections are often made at the two- to four-cell stage.

    Each animal blastomere receives at least one injection; however, multiple injections can be performed depending on the amount of mRNA needing to be delivered. Tissue-targeted injections can be performed as well based on fate maps provided on Xenbase.

12. Inject mRNAs using glass needles according to the following guidelines.
    - To quantify changes in microtubule dynamics, inject in vitro transcribed mRNA of fluorophore-tagged EB1 or MACF-43 (a truncated protein that contains a minimal EB1-binding domain [Honnappa et al. 2009]), at 300 pg/embryo or 75–100 pg/embryo, respectively (see Table 1).

      These mRNAs can be injected alongside overexpression or knockdown strategies for your protein of interest.

      Primary mRNA stocks are not diluted. The mRNA working concentration is generally set to 500 ng/μL. However, depending on the size of the construct, the working concentration can be higher in order to reduce the injection volume. Total injection volume is generally 3 nL per embryo. Both high concentration and high volume of mRNA can be toxic to the embryo. The concentration of mRNA of interest can be determined empirically depending on the construct and the purpose of the experiment. For example, for protein localization analysis the amount of mRNA should be titrated and the minimum amount of mRNA should be used to avoid overexpression artifacts, which can be elicited as protein aggregations.

    - To monitor changes in actin filament dynamics, use fluorophore-tagged actin binding domains such as LifeAct, Utrophin, F-Tractin, or actin monomer. Begin with LifeAct or Utrophin mRNA concentrations at 100–300 pg mRNA/embryo.

      It has been reported that these markers may impact actin dynamics and architecture in a dose-dependent manner (Belin et al. 2014; Flores et al. 2019; Legerstee et al. 2019). Therefore, take care to use the probe that will least impact the dynamic behavior of interest.

    - To assess cell–extracellular matrix adhesion dynamics, label focal adhesions with 50–75 pg/embryo of fluorophore-tagged Paxillin, Zyxin, or PAK2 DNA or mRNA.

      Many other labels are also possible (Kerstein et al. 2015; Stutchbury et al. 2017). Focal adhesion proteins have notoriously high cytoplasmic fluorescence, and TIRF microscopy greatly facilitates crisp adhesion segmentation in later analyses. Advanced imaging techniques such as fluorescence recovery after photobleaching (FRAP) can also be used to monitor the adhesion dynamics involved in cell migration and adhesion (Worth and Parsons 2010).

13. Upon completion of injections, transfer embryos into a dish containing 0.1× MMR.

14. Prior to incubation, sort out dead or unhealthy-looking embryos.

    Dead or unhealthy embryos can adversely affect the quality of healthy embryos. They be detected by the change in the pigmentation or embryo shape.

15. Incubate the embryos until they reach the desired developmental stage. To accelerate the rate of development, keep injected embryos that are stored in the 0.1× MMR-containing dish at warmer temperatures (20°C–23°C). Conversely, to slow development, keep embryos in cooler temperatures such as 16°C.

    The rate of embryo development is dependent on temperature. A chart for temperature-dependent development time can be found in Xenbase (https://www.xenbase.org/anatomy/alldev.do; https://www.xenbase.org/anatomy/static/xenopustimetemp.jsp).

    Axon outgrowth analysis is performed on neurons isolated from neural tubes harvested from embryos at stage 22. Embryos kept at 20°C–23°C reach stage 22 ∼24 h after fertilization. Once this stage has been achieved, proceed to Steps 16–23.

    A similar strategy can be applied to embryos to be used for neural crest cell isolation. Embryos kept at 20°C–23°C reach stage 18 ∼20 h after fertilization. Once this stage has been achieved, proceed to Steps 24–28.

Preparation of Neural Tube and Neural Crest Cell Cultures

For neural tube cultures, follow Steps 16–23; for neural crest cell cultures, follow Steps 24–28.

Neural Tube Culture

Isolation and preparation of neural tube cultures have been previously described in Lowery et al. (2012).

16. Before doing the dissections, prepare culture dishes as described in Steps 1–4. Fill the culture dishes with the neural tube explant culture medium.

17. Screen embryos with a fluorescence dissecting scope and identify which express fluorescence in the neural tube. Transfer these at stage 20–23 to an agarose-coated plastic dish containing Steinberg's medium.

    Embryos can display a mosaic of fluorescence owing to variable expression of the mRNA injected.

    It is possible, but more challenging, to dissect embryos beyond stage 23. The tissues adhere more tightly to one another, which necessitates longer collagenase treatment (in Step 19.i).

18. Isolate the entire dorsal portion of the embryo, including the neural tube, as follows.

    i. View the embryo with a dissecting scope and use fine forceps to remove the vitelline membrane.

    ii. While holding the embryo in place with one pair of forceps, use a second pair to create an incision on the side of the embryo, exposing the hollow interior.

    iii. Use both forceps to pinch along the tissue between the dorsal and ventral halves of the embryo, thereby cutting the embryo in half.

    Visual guidance for how to perform this step can be found in Lowery et al. (2012).

19. Isolate the neural tube as follows.

    i. Transfer the dorsal explant to a small tube or dish containing 2 mg/mL collagenase in Steinberg's medium and place on a rotator at room temperature for 15–20 min to loosen the tissues.

    ii. Pipette the explant into a plastic agarose-coated dish containing fresh Steinberg's medium.

    iii. Gently remove the neural tube from the dorsal epidermis and the ventral notochord using a pair of forceps. Insert the tip of the forceps in between the epidermis and the underlying tissue; then pull the epidermis back slowly to expose the neural tube. Hold the tissue with forceps and slide the tip of another pair of forceps between the notochord and neural tube.

    iv. Remove the somites on both sides using forceps.

20. Move the neural tube to a clean 35-mm dish containing plating culture medium for neural tube explants.

21. After collecting several neural tubes, transect each of them into approximately 20 thin slice explants using electrolytically sharpened tungsten wires.

22. Transfer the explants to the prepared culture dishes.

    The number of explants plated depends on the size of the culture dish used. To avoid overcrowding in the culture dish and to allow axons to grow freely, we generally plate 15–20 explants in each 35-mm culture dish and spread the explants evenly in rows (up to 10 rows) and columns (two columns). After plating, do not move the dishes, as this will disturb the attaching cells.

23. Allow explants to adhere for 12–18 h, ideally at ∼20°C–22°C.

    We typically leave the dish of cells on the bench at room temperature overnight. If conditions are appropriate, X. laevis neural explants send out neurites in a robust manner by 24 h after plating on the laminin/ poly-L-lysine substrate. Growth cones are highly motile on this substrate, extending outward from the explant in all directions, and the axons can achieve lengths of up to 1 mm (typical lengths are ≥100 µm). See Troubleshooting.

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Axons will extend radially, allowing various live imaging of growth cone behavior and cytoskeletal dynamics. Proceed to Step 29 to observe and image neurites and growth cones at room temperature 12–24 h after plating. RNA or plasmid products typically display variable expression between cells, so a range of fluorescence levels may be seen among growth cones. Expression of fluorescent protein RNAs can persist >48 h after plating, depending on the construct.

Cranial Neural Crest Cell Culture

A very helpful and thorough guide to neural crest isolation is given by Milet and Monsoro-Burq (2014), and we advise that this be heavily relied upon for tissue identification and dissection technique. We offer minor modifications here.

24. Prior to performing the dissections, prepare the culture dishes as described in Steps 5–10. Fill the culture dishes with plating culture medium for neural crest cells.

25. Sort embryos to identify those with fluorescence in the cranial neural crest region at stage 18. Strip vitelline membranes of stage 18 embryos, and embed them gently in a plasticine clay-coated Petri dish in plating culture medium for neural crest cells with the anterior dorsal regions exposed.

    Neural crest cells emerge from the tissue just along the anterior neural fold, which is raised slightly from the surrounding tissue.

26. Remove the skin above the neural crest using an eyelash knife. Apply gentle pressure along the edge of the neural fold to allow the neural crest (two to three cell layers) to separate. Lift the explant with a lateral/ventral flicking motion.

27. Rinse explants several times by gently pipetting into a fresh culture medium for neural crest cells, and then transfer the explants into rows along a fibronectin-coated imaging chamber.

    Explants should be at least 500 microns apart to ensure clear fields of view, but the exact number or density of explants in the chamber is not critical and will be dependent on size of the glass-bottom culture dish.

28. Allow the explants to adhere to the coverslip for at least 30 min in a cool (16°C–19°C) and vibration-free place before imaging (see Step 29).

    Tissue will begin collective cell migration within an hour of plating, and will subsequently delaminate to begin single-cell movement after 8 h or more, allowing for various measurements of cytoskeletal dynamics in both individual and collectively migrating cells. See Troubleshooting.

Imaging and Analysis of Cytoskeletal Dynamics

29. Once the cells are adhered, position the culture plate on the microscope stage. Perform live imaging of intracellular cytoskeletal dynamics in either neuronal growth cones or neural crest cells using spinning disk confocal microscopy, TIRF microscopy (for dynamics at or near the cell membrane), or even widefield fluorescence microscopy (for microtubule plus-end dynamics). While imaging, take the following points into consideration.

    - To analyze growth and pause events of microtubule plus-ends, which show very rapid dynamics, perform time-lapse imaging.

      Images should be captured at least every 2 sec for a duration of 1–2 min. Given the rapid dynamics of MT plus-ends, spinning-disk confocal microscopy allows fast and automated acquisition of large region of interests (ROIs) such as whole cells. It is incredibly important that laser power and exposure time of acquisitions are optimized to minimize light toxicity. Using short exposure times and low laser power with reasonably higher gain will help reduce phototoxicity and minimize photobleaching.

    - To visualize actin retrograde flow, use low-density labeling of fluorescent actin monomers followed by quantitative fluorescent microscopy or kymograph analysis (Watanabe and Mitchison 2002; Danuser and Waterman-Storer 2006; Yamashiro et al. 2014).

      Perform an initial test with 1- to 2-sec imaging intervals as a starting point, although flow rates will vary greatly based on structure stability. Focal adhesion dynamics of neural crest cells can be captured every 1–2 min for manual tracking of general size and turnover, but more sensitive tracking measures will often require much greater sampling rates, again within the order of 5–10 sec, for extended

imaging periods (20–30 min or longer). General surveys of cell morphology, perhaps to assess cell polarization or filopodial density/number following a genetic perturbation, can perhaps be reduced to every few minutes or collected at only select time points over a number of hours.

- If comparing dynamics of two labeled proteins, consider the time that passes while one is acquired and the system switches to capture the other.

  This is necessary because two fluorophore-labeled structures in separate imaging channels will not be captured simultaneously in typical single-camera/filter systems. The time that passes can be quite minimal with some setups (i.e., triggered acquisition using a small ROI) and dramatically delayed with others (i.e., averaged line scans on a large ROI). Ensure optimization of the microscope for rapid multichannel acquisition if this is of utmost importance.

- If cytoskeletal drugs are to be applied while imaging, administer treatment using a perfusion chamber.

  Many pharmacological agents are not easily soluble in aqueous media, and precipitation can occur if a concentrated solution is administered into a corner of the dish. Construct a perfusion chamber in the bottom of a typical inverted imaging chamber using vacuum grease and an additional coverslip.

  See Troubleshooting.

30. Process and analyze the collected images.

    - To quantify parameters of microtubule plus-end dynamics, use plusTipTracker, a Matlab-based open-source software package that automatically detects, tracks, and analyzes time-lapse movies of fluorophore-tagged cytoskeletal proteins.

      For a detailed explanation of how to manage files to perform microtubule dynamics analysis refer to Stout et al. (2014). It should be noted that a currently supported version of this pipeline has been revamped to analyze multiple types of dynamic particles and is now available under the name u-Track (Applegate et al. 2011).

    - Assess actin network flow using the quantitative fluorescent speckle microscopy (QFSM) technique and software (Mendoza et al. 2012).

    - Use appropriate pipelines for focal adhesion dynamics (FAAS) (Berginski and Gomez 2013), automated filopodia tracking (FiloQuant) (Jacquemet et al. 2017), and single-particle tracking (TrackMate) (Tinevez et al. 2017).

      Customized analysis methods can be built and streamlined in Fiji, Imaris, or Matlab, as preferred.

TROUBLESHOOTING

Problem (Steps 23 and 28): No neurite extension or cell adhesion is observed.

Solution: Neurites might fail to grow if the cultured explants did not adhere well to the culture plate. To ensure cell adherence, use freshly made culture medium and PLL and laminin-coated dishes for neural tube explants, and fibronectin-coated dishes for cranial neural crest. Try not to disturb the culture dish after explants are plated. Finally, it is possible that embryos used to establish the explant cultures were unhealthy, but because the embryos developed to the neural tube stage, this is unlikely to be the case. However, it is best to set aside some whole intact embryos from the same batch of eggs fertilized and used in the neural tube and neural crest cell cultures in 0.1× MMR along with dissections to ensure that continued development occurs.

Problem (Step 29): Cultured explants do not express the fluorophore-tagged protein.

Solution: One reason for low or absent expression is that the needle tip could be clogged depending on the injected material. For example, morpholinos are highly prone to clogging needles. To ensure a successful injection, occasionally check if the solution is coming out from the needle. Alternatively, it is possible that not enough material was injected. Optimal concentration will vary based

on transcript quality and molecular weight and should be titrated accordingly. Generally, the lowest possible concentration of a fluorophore-labeled protein is the most favorable one; low-level expression can achieve a much more favorable signal-to-noise ratio and minimize the artifacts caused by exogenous protein expression. Heavily overexpressed plus-end tracking proteins can be especially hard to deal with, as the excess fluorescent protein will coat the entire microtubule lattice instead of selectively labeling distal microtubule ends, rendering automated plus-end segmentation and tracking much more difficult.

Problem (Step 29): Growth cones or cells die during imaging.
Solution: Light toxicity is the major cause of the cell death. To avoid cell death, image samples at high gain rather than high laser power and keep exposure time short.

DISCUSSION

Xenopus laevis has been a powerful embryonic tool, not only for addressing questions regarding vertebrate development, but also for deciphering key cellular events. One advantage of *Xenopus laevis* as a model is that *Xenopus* embryos can tolerate extensive manipulation, and exogenous materials can be easily introduced via microinjections, such as fluorophore-tagged cytoskeletal-associated proteins. Compared to other systems, cell culturing methods are facile and do not require expensive culture equipment. Cultures can be generated in large quantities and maintained at the bench at room temperature.

The protocol described here takes advantage of this versatile model organism to examine the dynamics of the cytoskeletal components. Measures of cytoskeletal dynamics can be taken from any tissue of interest. To gather data from heterogeneous neuronal cultures, we use neural tube explants dissected from stage 20–23 embryos. Retinal cultures from embryos at stage 24 or beyond can also be used if a more homogenous neuronal population is desired. Cranial neural crest, a multipotent motile cell with well-established migration routes and fate determination cues, can be a valuable model for identifying effectors of collective cell migration, craniofacial development, and cancer metastasis.

The scope of the high-resolution live imaging of cytoskeletal dynamics in axonal growth cones and in cells can be expanded to study different aspects of the cytoskeletal behavior. By mimicking various physiological conditions, one can study how microtubule or actin dynamicity would change within the growth cone or cell in response to guidance signals introduced in culture medium. Multiple cues can be introduced to the cells to test the spatiotemporal changes in cytoskeletal dynamics, which could mimic the pathfinding behavior of the growth cone in its native environment.

RECIPES

Agarose-Coated Petri Dish

Dissolve 1% agarose in 0.1× Marc's modified Ringer (MMR) <R> solution and microwave to melt. Pour into a Petri dish until the dish is half-full. Let the agarose harden at room temperature. Store for up to 2 wk at 4°C.

Culture Media Base

Reagent	Amount	Final concentration
L-15 medium (Sigma-Aldrich L1518)	98 mL	49%
Ringer's solution (1×) <R>	100 mL	50%

Sterilize by vacuum filtration. Add 2 mL of Antibiotic-Antimycotic (100×; Gibco 15240062). Store at 4°C.

Marc's Modified Ringer's (MMR) (10×, pH 7.4)

Reagent	Quantity (for 1 L)	Final concentration (10×)
NaCl	58.440 g	1 M
KCl	1.491 g	20 mM
$MgSO_4$	1.204 g	10 mM
$CaCl_2$, dihydrate	2.940 g	20 mM
HEPES	11.915 g	50 mM
H_2O	to 1 L	

Adjust the pH to 7.4 using 10 M NaOH. Sterilize by autoclaving. Store at room temperature indefinitely.

Plasticine Clay-Coated Petri Dishes

To prepare dissection dishes, gather several 35-mm or 60-mm tissue-culture dishes (e.g., Falcon 353001). Push a small amount of plasticine clay (e.g., Plastilina Modeling Clay) into the middle of each dish, and then spread it evenly to fill the bottom 3–5 mm. Rinse the surface of the clay with ample water and ethanol to remove excess oily residue. UV-sterilize prior to use.

After the dissections, sterilize the dishes for reuse. Rinse them thoroughly with deionized H_2O to remove any embryo particulate, use a gloved hand to level out the depressions made for embryos, rinse with ethanol, and sterilize under UV light. Cap and store with dissection kit. Discard and make fresh dissection dishes if clay appears dirty or has trapped any debris.

Plating Culture Medium for Neural Crest Cells

Reagent	Amount	Final concentration
NaCl	1.55 g	53 mM
Na_2CO_3	0.265 g	5 mM
Potassium gluconate	0.527 g	4.5 mM
Sodium gluconate	3.82 g	35 mM
$MgSO_4$	0.060 g	1 mM
$CaCl_2$	0.055 g	1 mM

Add reagents to 400 mL of deionized (DI) H_2O. Adjust the pH to 8.0 with bicine, and bring to final volume (500 mL) with DI H_2O. Sterilize by filtration. Store frozen at −20°C in small aliquots. Add 50 µg/mL gentamicin sulfate and 1 µg/mL bovine serum albumin to the culture medium prior to use.

Plating Culture Medium for Neural Tube Explants

Reagent	Amount to add	Final concentration
Fetal bovine serum (Gibco 10438018)	20 µL	1%
Penicillin-Streptomycin (Sigma-Aldrich P4333)	20 µL	1%
BDNF (100 µg/mL; Sigma-Aldrich B3795; optional[a])	0.5 µL	25 ng/mL
NT3 (25 µg/mL; Sigma-Aldrich SRP3128; optional[a])	2 µL	25 ng/mL

Add culture media base <R> to a final volume of 2 mL (per 35-mm plate). Prepare plating culture medium fresh and maintain at 4°C.

[a]Axon outgrowth is greatly enhanced by addition of BDNF and NT3, but these may be omitted for guidance assays if cues will be added later.

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Ringer's Solution (10×)

Reagent	Amount	Final concentration
NaCl	33.6 g	1.15 M
KCl	0.93 g	25 mM
CaCl$_2$	1.11 g	20 mM
EDTA (0.5 M pH 8.0)	5 mL	5 mM

Adjust the pH to 7.4 and raise the volume to 500 mL with distilled water. Sterilize by autoclaving.
Store indefinitely at room temperature.

Steinberg's Medium

Reagent	Amount	Final concentration
NaCl	3.39 g	58 mM
KCl	0.05 g	0.67 mM
Ca(NO$_3$)$_2$	0.07 g	0.44 mM
MgSO$_4$	0.16 g	1.3 mM
Tris (1 M, pH 7.8)	2.3 mL	4.6 mM

Add reagents to 250 mL of deionized (DI) H$_2$O and adjust the pH to 7.8. Fill to final volume (500 mL)
with DI H$_2$O. Sterilize by autoclaving. Store indefinitely at room temperature.

Sylgard 184-Coated Petri Dish

Mix 10 parts polymer with 1 part curing agent. Pour the mix into a Petri dish until the
dish is half-full. Allow the mix to cure and harden overnight at room temperature. Store
indefinitely at room temperature.

ACKNOWLEDGMENTS

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