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Obtaining and maintaining primary cultures of neural crest-derived cells

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Heather Etchevers: This protocol was derived originally from those used by Drs. Elisabeth Dupin, Patrizia Cameron-Curry and Catherine Ziller, with the major goal of eliminating animal-derived feeder cells and chick embryo extract. See PMIDs: 1967835 (1990) and 8632985 (1996).;

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Abstract

A highly enriched population of neural crest cells (NCCs) from amniote embryos, such as from chicks, mice and humans, is desirable for testing the functional effects of molecular changes underlying numerous human diseases of neural crest derivatives and for investigating their potential for therapeutic compensation.

This protocol, adapted from the working protocol in our group over the last two decades, details the propagation of primary neural crest cells in a serum-containing medium. This medium can serve as a basis for comparison for more defined formulations in terms of maintaining multipotency and proliferation.

It is also conducive to the explant-based derivation and maintenance of neural crest-like cells differentiated from human induced pluripotent stem cells; melanocytes derived from primary congenital melanocytic nevi; satellite glia, neurons and Schwann cell precursors from mouse or human dorsal root ganglia; Schwann cells and their precursors and endoneurial fibroblasts from fetal human sciatic nerve; embryonic human, chick or mouse craniofacial mesenchyme; and human fetal sympathetic ganglionic cells.

Conditions favoring NCC expansion and the maintenance of their stem cell-like properties, as validated by immunofluorescence or RT-qPCR, are described here and in the originally published paper (**https://pubmed.ncbi.nlm.nih.gov/18689800/**) and protocol (**https://pubmed.ncbi.nlm.nih.gov/21959239/**). Although neural crest-like cells can be derived from a number of sites in the mature organism, full potential is best ensured by their purification from their source tissue at the outset of migration.

Attachments

PDF

Primary_NCC_cultures...

3MB

Guidelines

Primary cells are fragile. Use minimal time necessary to trypsinize, and similar to other stem cell types, find the happy medium between too dispersed and too confluent (**try to keep cells at about 50-75% confluence**. There will still be multipotent progenitors close to confluence although they are drowned out thereafter by larger, engaged/committed cells).

Freeze slowly but regularly at 0.5-1M cells/mL in 10% DMSO + NCC medium (>12% serum diluted by DMSO to about 10%) in an isopropyl alcohol bath at -80° for 3 hours. Then transfer as soon and quickly as possible to -150°C for long-term storage, either in an ultracold freezer or over liquid nitrogen vapors. Once frozen, DO NOT LET TUBE WARM ABOVE -80°C on pain of killing the cells, and a weekend is too long to leave them at -80°C, as they do start dying.

In contrast, **thaw quickly** from deep freeze by warming in a lukewarm waterbath or equivalent under agitation until a small ice fragment is still left in vial. Then, add briefly pre-warmed regular medium, and transfer all to a tube containing a volumetric excess of warm NCC medium before centrifugation and resuspension.

See Troubleshooting for further suggestions.

Materials

- Fetal calf serum, qualified for embryonic stem cells
- DMEM-F12 with Glutamax
- Penicillin/streptomycin concentrate 100X
- HEPES buffer, pH 7.4
- Hydrocortisone
- Transferrin
- T3 (3,3,5-thio-iodo-thyronine)
- Glucagon
- Insulin
- epidermal growth factor
- fibroblast growth factor 2
- Stericup 0.22 µm (Millipore)
- Rat tail tendon-derived liquid Collagen I (store 4°C and keep acid - warming or neutralizing will gel the stock)
- PBS
- BD Biocoat Collagen I-coated and standard tissue culture plastic 35 mm dishes as well as 25-27 cm^2 vented flasks

Troubleshooting

Problem

Contamination with non-neural ectoderm

Solution

Insufficiently close lateral dissection: Trim tissues closely and cleanly with the spring scissors for successful explantation. Contaminating non-neural ectoderm is easily removed with the neural tube, as it remains epithelial and keeps its integrity in vitro; however, as NCCs must migrate over it to reach the collagen, many NCCs may be removed along with the tube and ectoderm, thereby depleting cultures

Problem

Contamination with mesoderm

Solution

Insufficient separation of pancreatin-dissociated tissues: Visually inspect neural tubes to be explanted for any adherent mesenchyme and remove with forceps. If endoderm remains, there will surely be mesodermal contamination as well. Mesoderm is more white and opaque than the epithelial neural tube or the notochord; the endoderm is a sticky veil. For mouse NCC culture, tissues are stickier than those of human or chick after pancreatin treatment. If the application is for cell tracing after recombination with other cell types, in organotypic culture, in experimental chimeras30 or simply to determine if there is any contamination by non-NCCs, it may be useful to use the B6CBA-Tg(Wnt1-lacZ)206Amc/J mouse developed by the McMahon group and available from Jackson Laboratories. The Wnt1 promoter activates lacZ expression in all premigratory NCCs of the posterior cephalic, vagal and trunk lineages. Other lineage reporter lines are available; fluorescence is more sensitive.

Problem

Heterogeneity; unknown cells in mixed culture

Solution

Enrich early primary NCC using CD271-conjugated magnetic beads and passing the detached cells during the second passage over a MicroMACS column (Miltenyi; https://www.miltenyibiotec.com/UN-en/products/cd271-microbead-kits-human.html#130-099-023). We have used the MS columns (https://www.miltenyibiotec.com/UN-en/products/ms-columns.html#130-042-201) in the past. Choose the species-appropriate product. This is the best technique in our experience as it does not subject cells to the extreme shear and pressure stresses involved in FACS. However, pre-conjugated bead sorting is only possible for human and mouse cells (and other species recognized by conjugated CD271/NGFR antibodies). Another solution for avian embryonic neural crest, and possibly for human, would be to pre-incubate cells briefly with the HNK1 antibody (https://dshb.biology.uiowa.edu/HNK-1) and then sorting with beads against mouse IgM. These are not available from Miltenyi; only human IgM at the time of writing.

Problem

Neural tube wicks to side of 35-mm dish upon transfer to incubator

Solution

Liquid sloshing pushed the tube from under the meniscus to the edge: It IS possible to replace the tube in the middle of the dish using the same gesture without damage. Keep horizontal during the transfer to incubator and move slowly and mindfully.

Problem

Few or no NCCs migrate

Solution

The explants may be too dry: If the air circulation does not bring enough humidity to the early explants, place each 35-mm dish in a 10-cm dish (or perhaps many into a larger, clean recipient), with sterile, wet gauze in the larger plate, before incubating. The staging may not be appropriate for the neural tube level explanted: Dissect a more caudal piece of neural tube or the same level but from a younger embryo. The collagen substrate is uneven on the culture plastic: Neural crest cells migrate adequately on collagen I–coated plates. Reduce variability in coating by using commercially produced coated 35-mm plates for explantation; for precious cell cultures, we continue to use commercial ware for subsequent passages as well. The neural tube was not in close apposition to the plastic the first night and did not adhere: Ensure that the neural tube does not detach upon addition of medium on day 2. Empirically, hundreds of NCCs migrate away from avian neural tubes (chick or quail), while human and mouse neural tubes yield 60–150 cells for equivalent-length fragments

Problem

Trypsinization damages cells

Solution

Adherent chicken, mouse and human NCCs all secrete other extracellular matrix factors. If the cells are approaching confluence, they can sometimes be refractory to detachment after trypsin treatment. Passage cells at a lower density. Aliquot trypsin-EDTA by 10 ml, freeze at −20 °C and use within a week of thawing, or use the trypsin-like enzyme in the TrypLE-Express formulation by Invitrogen (cat. no. 12604-013), which remains active after repeated heat-cool cycles.

Problem

Cells do not proliferate adequately

Solution

Other brands of culture media and adjuvants have worked well if the catalog references used here are unavailable. Supplement aliquots of basic medium (DMEM and F12) and use within 2 weeks; otherwise the growth factors may no longer be bioactive. Cell cultures should be checked for bacterial or yeast contamination, and if the problem persists, then mycoplasma may also be an issue. A standard PCR-based test should yield results quickly. In our hands, this has never been a problem, but we check cultures for mycoplasma at each freeze and thaw regularly in the facility. Serum lots vary and should be tested. We have

successfully tested multiple lots for chicken NCC proliferation and survival for later use with human cells. Serum substitutes (eg. KnockOut Serum Replacement) have not given satisfactory results to date; they do often promote survival but, on occasion, differentiation. Some cells change morphology over time even starting with an apparently homogeneous culture, with the majority favoring an elongated, thin spindle shape or a large, stellate form with visible polymerized actin fibers under phase-contrast illumination. These cells will continue to proliferate for some time but will senesce after a certain number of passages. However, if all the stem cells have been depleted, the entire culture will still survive for months, even at low cell density, which also favors senescence. Proliferating NCC are small tripolar or multipolar cells with convex edges and filipodia at the corners; refringent bipolar cells are gliopigmentary progenitors; the larger senescent cells may be mature myofibroblasts as a default outcome.

Safety warnings

FGF2 is quite labile at 37°C (half-life of around 2 hours, reportedly). Aliquot stock medium after making it up in order to pre-warm as minimally as possible small amounts at a time, such as 10 or 25 mL, according to consumption.

Before start

Prepare the following stock solutions:

Hydrocortisone: Resuspend 1 mg with 1 mL EtOH 100%. After dissolution, add 19 mL sterile water for stock @ 50 µg/mL. Label "HC", aliquot by 200 µl, keep @ -20°C.

Transferrin: Resuspend 100 mg with 10 mL sterile water for stock @ 10mg/mL. Label "X", aliquot by 100µl (or 1mL), keep at -20°C (working) or -80°C (long-term).

"T3" (3,3,5-thio-iodo-thyronine): Resuspend 1 mg with 1 mL of NaOH 1M. Mix, then add 49 mL sterile water (**stock = 20 µg/mL**). Aliquot 4 × 12 mL and keep @ -80°C.
To 1 mL of this stock, add 9 mL sterile water to get 10 mL **1Xsolution @ 2 µg/mL**. Aliquot by 20 µl (and 2 mL for the rest).

Glucagon: Resuspend 2 mg with 4 mL acetic acid 1M (285 µl glacial acetic acid for 5 mL sterile water) for **stock solution A @ 500 µg/mL.**
10 µl of stock A labeled "Glc A" and 990 µl sterile water for **stock solution "Glc B" @ 50 µg/mL**.
Aliquot stock A 4 × 1 mL; stock B by 20µl and store @ -80°C. 10 µl of stock B diluted with 990 µl sterile water will yield 1 mL "Glc **1X" solution @ 50 ng/mL**. Aliquot by 40-50 µL, can store @ -20°C.

EGF: Resuspend 100 µg with 10 mL PBS (no Ca++ / Mg++, optimally with 1% BSA, but ok without) for **1X solution @ 10 µg/mL**. Or keep 50 µL aliquots of a 200 ng/mL first stock solution at -20°C.
Aliquot 10 µg/mL solution by 10 µL and store @ -80°C.

Insulin: Resuspend 100mg in 9.9 mL sterile water with 100µl glacial acetic acid, for **stock solution "Ins A" @ 10 mg/mL**. Dilute 10 µl into 990 µl sterile water for 1 mL **stock solution "Ins B" @ 100 µg/mL**.
Aliquot stock A in lots of small aliquots if possible and keep @ -80°C.
Dilute 200 µl stock B in 1.8 mL sterile water to yield 2 mL **@ 10 µg/mL, and aliquot "Ins 1X" solution** by 10 µl. Can keep these aliquots, to be used within 6 months, @ -20°C.
[Or : From a 5 mg/mL stock, take 2 µl and dilute to 1 mL = 10 µg/mL, aliquot as B above.]

FGF2: Resuspend 25 µg with 900 µl PBS and 100 µL serum, or 1% w/v BSA, for a stock solution @ 25 µg/mL.
Dilute 20 µl with 180 µl de PBS or serum-containing medium. Aliquot these 200 µl by 10 µl and the stock by 100µl, keep all @ -80°C. Can be diluted to 10 µg/mL (4X) as stock if easier.

Coat culture supports

1 If using #1 or #1.5 glass coverslips in wells for microscopy (eg. 12mm diameter in 24-well plates, though **these** also look appealing), pre-coat to confer a positive charge with **poly-D(or L)-lysine**. Polylysine is dissolved in sterile ultra-pure water (20 to[preferred] 50 µg/ml at pH 6-7) and adsorbed on coverslips for 1h or more. Add 0.15 mL/cm² or enough 0.5% w/v solution to cover culture surface and incubate at room temperature. Remove and rinse 1x in water or PBS.

2 Stock collagen is at (or can be diluted to) 1 mg/mL in 0.01-0.1 M acetic acid (Sigma #C8919).

3 Extemporaneously dilute collagen in 1X PBS to 50 µg/mL ready for use = 2.5 mL e.g. in 50 mL. This should neutralize acid and start polymerizing. Possible to do in 1X medium with phenol red to visualize pH neutralization.

4 Leave collagen solution on warm plate surface for 2h (in cell culture hood or incubator) to finish polymerization.

5 Aspirate off liquid and rinse 2x in 1x PBS (in an ideal world) or media. Add media to keep collagen moist.

6 Add cells as usual. Vessel can be kept in cell culture incubator with liquid still on, or aspirated and dried, or kept with PBS or medium to remain hydrated with the edges wrapped in Parafilm at 4°C. All seem to work at least sometimes.

Effective medium for primary cultures of neural crest cells (not animal origin-free!)

7 1. For 200 mL total medium, put the following in the top compartment of a 150-250 mL 0.22 µm membrane filtration unit (eg. ref **051250B** from Dutscher; Millipore Steritop GV with PVDF membrane 0.2 µm is the best for low absorption of growth factors):

7.1 170 mL / 85.0 mL DMEM-F12 with Glutamax (or 68 mL DMEM + 100 mL F12, 2 mL glutamine/Glutamax) for a final concentration of 85% + 1% (2mM) total glutamine equivalent

7.2 25 mL fetal calf serum (prefer ES-qualified or for primary human cells, *eg*. PromoCell, cat. no. C-37355) for a final concentration of 12.5% (12% works also)

7.3 2 mL penicillin/streptomycin 100x for 1% (100 U/mL penicillin; 0.1 mL/mL streptomycin)

7.4 2 mL HEPES buffer 1M for a final concentration of 1% (10 mM).

7.5 50 µL hydrocortisone (HC) @ 0.4 mg/mL for a final amount (concentration) of 20 µg (0,1 µg/mL).

7.6 200 µL transferrin (“X”) @ 10 mg/mL from 1 mL aliquots, can be opened and stored at 4°C for 2 months, for a final amount (concentration) of 2 mg (10 µg/mL).

7.7 40 µL T3 @ 2 µg/mL for a final amount (concentration) of 80 ng (0.4 ng/mL).

7.8 40 µL glucagon (“Gc”) @ 50 ng/mL for a final amount (concentration) of 2 ng (10 pg/mL).

7.9 20 µL insulin (“I”) @ 10 µg/mL. Stock can remain at 4°C for a month. For a final amount (concentration) of 200 ng (1 ng/mL).

7.10 Add 2 µL EGF at 10 µg/mL, which can be opened and stored at 4°C for 2 months though is optimally kept at -80° minimizing freeze-thaw cycles. This is for a final amount (concentration) of 20 ng (0.1 ng/mL). Cultures appear to tolerate ten times as much EGF without visibly altering their properties but this remains to be tested rigorously.

7.11 **Filter sterilize in a 150 mL Stericup 0.22 µm from Millipore (ref 51244 Dutscher ; GV better to not adsorb growth factors).**

7.12 Add 16 µL sterile FGF2 at 2.5 µg/mL, kept at -80°C in small aliquots so as to not freeze/thaw multiple times. This is for a final amount of 40 ng or concentration of 0.2 ng/mL.

8 Keeps OK up to 6 weeks at 4°C but best to use within 2 weeks.

9 Primary mouse, chick and human NCC grow well on BD Biocoat Collagen I e.g. ref 356456 or 354456 esp. at beginning, but then even ordinary tissue culture-type charged plastic as a T25-T75 is OK. The NCC make their own matrix.

Explanting neural crest-containing tissues - considerations

10 Our group developed the protocol described here in order to study the transcriptome of human primary pluripotent NCC cultures, with the idea of favoring a simple, inexpensive matrix if possible, and of defining the medium somewhat more so that it would not contain chick embryo extract or leukemia inhibitory factor (a common adjuvant for mouse stem cells but unnecessary for pluripotency in human embryonic stem cells).

Small adjustments to the culture medium and the absence of irradiated fibroblast feeder cells are the major differences with similar protocols. The self-renewing potential was unexpected (we kept line N5 cycling for 9 months, and have frozen and thawed it and other lines many times).
Empirically, we have found this protocol to be equally applicable to the derivation and maintenance of avian, rodent and human NCCs, permitting a laboratory that acquires expertise in isolating cell lines from one species to apply the technique for interspecies compaisons, the analysis of experimental chimeras, or for explanting any Schwann cell progenitor-containing (ie. innervated) tissue, particularly fetal.

In brief, the technique involves microdissection and isolation of a length of embryonic neural tube at stages at or preceding the period of NCC emigration from the desired level. The neural tube is placed on a collagen I–coated tissue culture dish under a meniscus of medium to ensure adhesion and maximal contact, and then fully submerged in a medium that favors proliferation of undifferentiated cells to enable NCCs to migrate away from the neural tube. The tube is removed with a custom-shaped sterile glass tool, the cells detached and re-seeded at a regular and somewhat low density into a new collagen I–coated plate. These cells multiply vigorously and are then available for further passages, freezing and experimentation.

11 **Pancreatin preparation and optimization.** Make up at 25 mg/ml in 50 ml PBS, dissolve at room temperature (20–23 °C) overnight with agitation, centrifuge the remaining deposit, filter-sterilize and aliquot by 1 ml. Store at −20 °C.

After thawing, recentrifuge in a microcentrifuge at maximum speed, then dilute te supernatant with three
parts warmed PBS (37 °C) for use.

Do not keep diluted pancreatin.Because powdered pancreatin is not entirely soluble, there will be variability in lot activity after filtration. A sufficient volume of stock solution should be prepared and divided into 1-ml aliquots at least the day before, and frozen so as to minimize variability from one tube to the next. Digestion that loosens epithelia before dissociating different tissue layers from one another is too harsh and more PBS should be used to dilute the stock; optimal digestion occurs in >3 but <10min. Pancreatin lots should be tested in advance: when the somites fall out intact from between ectoderm and endoderm upon gentle agitation by the forceps, the pancreatin concentration is optimal.

12 Try a concentration of pancreatin (6 mg/mL, Sigma-Aldrich, cat. no. P3292) diluted in Ca++/Mg++-free PBS. To stop digestion, immerse tissue piece in DMEM or RPMI with 12% (vol/vol) FCS. Keep as long as it is sterile. Better if the pH is controlled around 7.4 with bicarbonate and/or HEPES as per the complete medium.

Microdissection

13 **Pulled Pasteur pipettes.** These should be prepared just before use to keep sterile and unbroken. When it is necessary to remove the cleaned neural tube from the culture dish, grasp the cotton end of a pipette in one hand and the tip in the other, and hold the pipette over a flame at about 4 cm proximal to the tip, rotating to heat all sides. As the glass reddens, remove from flame, quickly pull about 40 cm apart to make a thin thread, let cool for an instant, and then break by bringing hands closer in a sharp movement. Bring the tip from the larger part back into flame for 1 s, to round and seal. Place it immediately into the laminar flow hood and let it cool without touching the tip to a surface. Take the necessary precautions for operating an open flame; work away from inflammable objects and currents in the hood or on benchtop.

14 Multiple chick, mouse or human embryos can be microdissected away from their annexes and kept in PBS or RPMI on ice for up to 2 h with no adverse effects. The same for sciatic nerves, dorsal root ganglia, or craniofacial mesenchyme.

15 Use a glass Pasteur pipette, having wet in protein-containing medium, to transfer segments of neural tube cut away from most surrounding tissues into 22–37 °C prepared pancreatin in a 35-mm dish.

16 Allow segments to incubate at room temperature for 6–7 min. Monitor digestion carefully. With experience, the timing is very reproducible at a given stage, species and level of neural tube. The ectoderm may appear as a thin veil that is detaching from the somites and can be teased away. When the neural tube becomes slightly wavy, it is time to slow the reaction by removing the tubes to clean PBS with minimal transfer of the enzyme solution; use the same Pasteur pipette.

17 Quickly tease away the sticky lateral tissues with fine forceps **without directly touching the neural tube** so as not to damage the epithelial integrity of the desired tissue. Peel away the veil of endoderm, then
the ectoderm. Lateral movements along the tube (holding the mesoderm, or an excess length of neural tube) will detach somites or pharyngeal and head mesoderm easily to yield a clean neural tube.
Finally, it is possible to grasp an end of the notochord and separate it from the neural tube, which may be maintained against a forcep tip but not pinched between them, thereby carrying some paraxial mesoderm with it away from the tube.

18 Transfer cleaned neural tubes immediately to 1 ml of complete NCC medium in a 35-mm dish to stop digestion and allow tissues to recover. Cleaned neural tubes can be kept for up to 1 h in enzymatic stop medium at room temperature before transfer to culture dishes.

19 In the tissue culture hood, place **315 µL** of sterile, complete medium into 35-mm collagen dishes or six-well plates (one dish/well per explant). Wet the entire surface, but do not

scrape collagen with the pipette tip so as to maintain optimal adhesion and outgrowth conditions.

20 Bring the neural tubes from Step 18 into hood. Use a fire-polished Pasteur pipette (better than a plastic pipette tip) to carefully draw one neural tube into the opening with some medium. Let the tube fall to the bottom of the liquid within the pipette by gently moving up and down within the thin portion until it
abuts the lower meniscus.

To allow the tube to be wicked out onto the dish with **minimum addition of complete medium**, gently appose the tip at a 45° angle to the center of a moistened dish, apply pressure, and draw the tip across a few millimeters with a slight lifting movement. The neural tube will flatten and be pinned under the meniscus at the air-liquid interface. Allow a few microliters of liquid to be transferred with the neural tube or it will stretch and break.

21 Replace the cover of the dish and place it in an incubator at 37 °C, 5% CO_2 overnight. Make sure the incubator is saturated in humidity.

22 The following morning, warm a 10-ml aliquot of complete medium for 20 minutes.

23 Slowly add 1 ml of medium to each explant by first gently moistening around the adherent neural tube; avoid a strong jet. It is essential that explants remain submerged and attached to the substrate or no NCCs will migrate onto the culture dish.

24 Replace the dishes in an incubator and allow the cells to migrate on undisturbed plates for 18-24 hours.

25 Remove the lid from the culture dish and, by using a freshly prepared pulled glass pipette with a rounded tip (a plastic pipette tip will do at a pinch but is not as tidy), push at one end of the adherent neural tube as if gathering the tissue into the center. It should detach readily from the surface, along with any adjacent ectoderm, without fragmentation. Push inwards from the other end as well until detached.

Tease these epithelia and stray floating debris away from the mesenchymal cells and aspirate the medium, replacing it with fresh, complete medium prewarmed to 37 °C. Verify under the inverted microscope that no pieces either remain in the center of the explants or float in the medium, as they will reattach and spread.

26 Return the dish to the incubator. Cells may be dissociated and reseeded at this stage, although we typically see better survival rates if we allow them to continue proliferating for an additional day before the
first passage.

27 To passage cells, remove the medium by aspiration, rinse the culture with sterile PBS prewarmed to 37 °C, and add 0.5 mL (for 35-mm dishes) or 1 mL (for 10-cm dishes) of trypsin-EDTA to the dishes. Return dishes to the incubator for **3 min**. A gentle, lateral tap should show most cells to have detached when examined under the inverted microscope. Do not incubate for more than 5 min so as to conserve cell viability.

28 Cells will need to be passaged at 1:3 every 2–3 d. They must not be more than 70% confluent unless spontaneous differentiation and arrest of proliferation is desired. Nonetheless, even in highly confluent
cultures, some highly proliferative cells often persist and can be amplified again at a slightly lower density.

29 Use pipette to add 0.5 or 1 ml of PBS prewarmed to 37 °C so that the jet of liquid copletes cell detachment. Avoid vigorous up-and-down shearing movements of liquid, or bubbles that can damage and kill cells.

30 Transfer the liquid to a 15-ml conical centrifuge tube, add 10 mL of enzymatic stop medium or PBS at a pinch to dilute, and spin at room temperature at 1,100*g* for 5 min. The NCC themselves are small so need this greater force to settle.

31 Aspirate the supernatant and gently resuspend the pellet in complete medium with an appropriate volume for the new matrix-coated vessel, using a fire-polished Pasteur pipette to avoid shearing.

32 Cells may be stored at 5×10^5 cells/mL of cold freezing medium (10% DMSO or commercial media for freezing pluripotent stem cells both work). Freeze cryotubes progressively in an isopropanol-filled container at −20 °C for 2 h, followed by 3 h to overnight at −80 °C (no more than 18 h) and long-term banking over liquid nitrogen vapors or at -150°C in an ultracryofreezer.

33 Thaw cells according to standard procedures with a rapid warm-up at 37 °C, immediate transfer while some ice still persists to 10 mL of pre-warmed complete medium, centrifugation (as per Step 30) and resuspension of the pellet in fresh medium. Seeding density for regrowth can be anywhere from 2,000–6,000 cells/cm^2.

Protocol references

Protocol adapted from:

Etchevers, H. Primary culture of chick, mouse or human neural crest cells. *Nat Protoc* **6**, 1568–1577 (2011). https://doi.org/10.1038/nprot.2011.398

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