Isolation and Culture of Postnatal Rat Gut Neural Crest Stem Cells (NCSCs)

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Postnatal rat gut neural crest stem cell (NCSC) isolation and culture

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Postnatal gut NCSCs can be isolated by flow cytometry and cultured in essentially the same manner as those isolated from E14 sciatic nerve or gut. However, differentiation medium (added after the first 6 days of culture) should be supplemented additionally with 5% fetal bovine serum. Preparation of media, tissue culture plates, and staining medium is the same as for fetal NCSCs, but repeated here for convenience.


1. Preparation of the 'standard' culture medium and plates prior to cell isolation

A. Self-renewal medium for NCSCs

Self-renewal medium (typically used for initial plating of cells):

To make 100 ml of medium: 
- DMEM-low glucose, 80 ml
- Chick Embryo Extract (CEE) 15 ml
- Penn/Strep (P/S) 1 ml
- N2, 1 ml
- B27, 2 ml
- Retinoic Acid (RA; 117 μM stock = 35μg/ml), 100 ul
- 2-mercaptoethanol (2ME; 50 mM stock), 100 ul
- bFGF (25μg/ml stock), 40 ul
- IGF-1 (20 ng/ml of media)

B. Differentiation medium

Refeeding medium for differentiation (typically added after 6 days of culture):

To make 100 ml of medium: 
- DMEM-low, 95 ml
- Chick Embryo Extract (CEE) 1 ml
- Penn/Strep (P/S) 1 ml
- N2, 1 ml
- B27, 2 ml
- Retinoic Acid (RA; 100 μM stock), 100 ul
- 2-mercaptoethanol (2ME; 50 mM stock), 100 ul
- bFGF (25μg/ml stock), 40 ul
- FBS 5 ml
Write out the recipe each time the experiment is performed and check off the ingredients as you go. The cells are very sensitive to alterations in culture conditions. If you are careful to keep a checklist of medium components you will avoid errors in medium preparation and will be able to rule out errors in the event of culture failure. When preparing the medium, put half of the DMEM-low in the 0.22 um filter unit, add the rest of the ingredients, and apply vacuum to the filter. After drawing the medium through the filtration unit, put the rest of the DMEM-low through the filter. Do not freeze/thaw anything more than twice. Add the 2ME first and pipette up and down to disperse it completely. It is a strong reducing agent. Add the RA second and pipette to disperse. RA is dissolved in ethanol. Before dilution, both 2ME and RA can denature proteins in the medium. Make sure the CEE, N2, B27, and P/S stocks are resuspended well after thawing. The 2ME and RA can be freeze/thawed as many times as needed. Adding 5% FBS to the differentiation medium enhances neuronal and myofibroblast differentiation of postnatal gut NCSCs. However, be careful not to add it to embryonic NCSC culture, as it promotes smooth muscle differentiation often to the exclusion of other cell fates.

C. FACS buffer/staining medium

After the cells have been dissociated, they are maintained on ice in "staining medium" that is prepared as follows:

FACS buffer for neural cells (150ml):
- L15 medium without phenol red (132 ml)
- 1% penn/strep (1.5 ml, 10,000 U/ml stock)
- 1mg/ml BSA (150 mg, tissue culture grade)
- 10mM HEPES pH 7.4 (1.5 ml of a 1M stock)
- 10% Biowhittaker water (15 ml)

After cells have been dissociated they should be kept in FACS buffer and on ice.

D. Preparation of tissue culture plates

If NCSCs are cultured as neurospheres, they are grown under non-adherent conditions in ultra low-binding plastic plates. If NCSCs are cultured in adherent cultures, the plates are coated with PDL and fibronectin as described below.

To prepare the plates, thaw a vial of Poly-D-Lysine (PDL; 150μg/ml). PDL (Biomedical Technologies) arrives lyophilized and should be reconstituted in sterile water. It is stored frozen (-20 C) and thawed as many times as necessary. Coat each well with enough PDL to cover the bottom of the well, then transfer to the next well. Do this until all the wells have been coated with PDL. Allow the plates to dry in the hood. Keep the PDL to reuse again and again. After the wells are dry, rinse with Biowhittaker water, and aspirate. Allow the plates to dry in the hood. When dry, coat with fibronectin (Biomedical Technologies) that was dissolved overnight at 37°C at a concentration of 1mg in 6ml (see below). Store unused fibronectin solution in the refrigerator, but do not re-use for NCSC culture. The medium should be made before coating the wells with fibronectin, and make sure not to let the fibronectin coat dry before adding the medium. Add 1.5mL of medium per well of 6 well plates, 1.0 ml/well in a 12 well, 0.5 ml/well in a 48 well and 100 ul/well in a 96 well. Store the plates in the incubator until the cells are ready to be plated. It is important not to let the plates equilibrate with the air because the medium is buffered by bicarbonate and will become bright pink (highly basic pH and toxic to cells). If cells are to be plated directly at the flow cytometry facility, plates are placed inside open ziplock bags inside the incubator and then sealed just before transport. Prepare the fibronectin the day before by adding 0.5 ml of Biowhittaker water to a 1 mg vial of human fibronectin. Incubate for one hour at 37°C so that the fibronectin may dissolve. Do not vortex, or pipet to aid dissolving. After an hour, bring to volume with 5.5 ml of DPBS and store in the incubator overnight. After the initial use, date the fibronectin vial and store in the refrigerator. It may be used for other cell types but the vial will not be used again for NCSC. Store up to two weeks.

2. Dissection of myenteric plexus and muscle layers from gut Epithelium

Fill several petri dishes with sterile HBSS free (no calcium or magnesium). These will hold the guts after they have been dissected out. Place the dishes on ice to keep the tissue cold. Reserve one petri dish per sample with HBSS free to hold the pieces of the outer muscle layers that have been dissected off of the guts. Also, prepare 15 ml centrifuge tubes for each sample with 4 ml HBSS free and place on ice. These will hold the dissected muscle layers after they are minced.

Fill the bottom of the dessicator chamber with crushed dry ice. Replace the ceramic plate and cover it with a layer of paper towels. Place rats in the dessicator chamber, and wait 3-4 minutes until all breathing and movements have stopped. Assure that the animals are dead by dislocating the neck or cutting a pneumothorax. Wet the abdomen with 70% alcohol and cut through the skin and abdominal muscles to reveal the abdominal cavity. Pull the gut out gently with a smooth motion using the tweezers. This will cause the mesentery to pull off and be left behind in the animal. Peeling the muscle layers off is much easier once the mesentery is removed. However, for very early postnatal
animals (P5 or younger), this procedure may pull off much of the muscle layers as well. In that case, you may want to carefully cut the whole gut out and remove the mesentery under a dissecting microscope. In general, only take the gut distal to the stomach and proximal to the large intestine, but NCSCs can also be isolated from those segments if desired. Puncturing the large intestine may release large amounts of bacteria. Place either whole gut (P22 or earlier) or 3-4 inch pieces of gut into the dishes containing cold HBSS free.

Use a dissecting scope and Moria or Dumont forceps to peel the outer circular muscle layers from the gut. These extend around the entire circumference in the intact animal, but pulling the guts free of the mesentery usually results in the loss of the muscle layers on one side. In general, you will see a white translucent strip somewhat loosely adhering to the gut epithelium. This can be peeled off like a banana. However, it usually adheres much more tightly in adult animals and may need to be teased away from the epithelium. Collect all peeled muscle layers in a clean dish of HBSS free on ice as you dissect. When all animals of a particular sample are finished, mince the pieces of muscle in HBSS free with scissors as finely as possible. The pieces should be small enough that they can easily be drawn up into a P-1000 pipette tip. Transfer the minced gut pieces to the prepared centrifuge tubes.

3. Dissociation of the cells

The tubes of minced tissue can remain on ice until all samples are ready to dissociate. Then spin down the cells at 1000 rpm (210 RCF) for 4 minutes while preparing the dissociation medium. Usually 2-4 ml per sample is adequate. For 2 ml, use 800 μl HBSS free, 1 ml trypsin (0.05% trypsin-EDTA, Gibco), and 200 μl collagenase IV (Worthington Biochemicals, 10 mg/ml dissolved in HBSS with calcium and magnesium, stored in aliquots at -20 C). Discard any unused enzyme. Remove HBSS free from spun minced tissue and add dissociation medium. Add vigorously to disperse pellet, but don't triturate at this time. Dissociation time varies with the age of gut. P5-P22 gut can be dissociated for 20 minutes at 37°C with agitation every 5 minutes to prevent settling of tissue. Gut older than P22 should be dissociated for one hour and requires more vigorous mechanical dissociation (may also increase yield of younger gut cells). Every 5 minutes, strongly flick tube in a downward, outward motion away from the body. That is, hold tube near the top and flick contents as strongly as you can down into the bottom of the tube. Your arm may become sore. While incubating, prepare quench solution. Add twice as much quench to each tube as dissociation medium was originally added. For 4 ml, use 4 ml staining medium/FACS buffer and 30 μl of DNAse I (Sigma, 5 mg/ml stock in HBSS free, aliquot and store in -20 C). Make sure the aliquot of DNAse has not been previously thawed, and discard after use. When incubation is complete, add quench to all samples. Flick vigorously to loosen cells from the remaining pieces of tissue and distribute DNAse. Place samples on ice. Make sure samples are cold before centrifuging again (210 rcf, 4 minutes). Resuspend in 1 ml of staining medium. Pipette cells vigorously being careful to disperse pellet after beginning to pipette. Filter each sample through a piece of nylon mesh (45 microns, Sefar America) and divide among the FACS tubes (Falcon 352058).

4. Staining the cells for flow cytometry

Compensation tubes (these tubes will be used to set up the FACS machine):
1. Be sure to reserve some sample that will be stained with 7AAD only to gate out the dead cells. The 7AAD (Molecular Probes) stock is made in water (1 mg/ml). The stock should then be diluted 1:500 in staining medium for use. All samples are ultimately resuspended in this solution after antibody staining is complete, usually 500 ul.
2. You will need one compensation tube stained with a single fluorochrome if you are using multiple fluorochromes on your sample (e.g. one FITC and one PE). All antibody incubations should be done on ice for 20 minutes. Usually 100-200 μl of antibody solution per tube is sufficient. Rinse antibodies off by filling the FACS tube 2/3 full with staining medium at the completion of incubation and spin down. Cells can then be resuspended in the next antibody or in 7AAD if they are finished. All spins are at 1000 rpm for 4 minutes at 4°C as used for dissociation. Postnatal NCSCs can be identified as those expressing the highest levels of p75 staining. P75 (I192g, Oncogene) and alpha4 integrin (BD Pharmingen) have the same isotype (mouse IgG1), thus they can only be used together if one of these antibodies is directly conjugated to a fluorochrome. For example, stain with the unconjugated p75 antibody (1:200) first, then anti mouse IgG1 FITC (Southern Biotechnology, 1:200), and then the directly conjugated anti-alpha4. If both antibodies are conjugated they can be used directly to stain the cells in one step. For primary antibodies, multiple dilutions should be tested on the first use and then the most dilute solution that gives acceptable staining should be used from thereafter.
Below are some representative FACS plots with gates drawn around the populations where NCSCs are highly enriched. Also, you may want to consult Cell 96:737 and Neuron 35:643. If the cells are sorted directly into culture, be sure to equilibrate the culture plates in 6.5% CO₂ incubators prior to sorting. While preparing to sort the cells, the plates can be stored in sealed ziplock plastic bags. The sort should be performed as quickly as possible to avoid allowing the culture medium to equilibrate with air (don't allow the plates to remain open to the air long enough to get pink). After the sort, the plates can be placed back into baggies, and gassed with 6% CO₂ to restore proper pH balance while waiting to go into incubators.

5. Culturing the cells

A. Culturing the cells at clonal density

Progenitors are cultured at clonal density so that individual NCSCs can form spatially distinct colonies. This allows us to monitor the proliferation, self-renewal, and differentiation of individual NCSCs. This is different from the mass cultures used in other laboratories, where many different progenitors contribute to the growth of the cultures, but where the relative contribution of individual progenitor cells to the proliferation or differentiation within the cultures is uncertain. By performing clonal analysis heterogeneity among progenitors can be directly assessed. Clonal density means that NCSCs are plated at a very low density: typically 40 NCSCs are added per 35 mm dish (embryonic through P22 gut NCSCs), resulting in the formation of 10-20 colonies. At this density, individual colonies are still well separated even after proliferating for 2 weeks. This allows the lineage compositions of individual clones to be determined (by immunofluorescence staining of lineage markers), and compared. A larger number of cells should be plated per well of adult NCSCs (~150 p75high cells) since it is not yet possible to obtain a pure population of these cells by flow cytometry. In addition, adult NCSC are typically smaller than embryonic NCSCs making it less likely for colonies to mesh together.

B. Refeeding cultures

Under standard conditions, cells are cultured for 6 days in standard medium (see 1A above), then switched to a similar differentiation medium (see 1B above) for another 8 days before immunohistochemical analysis of colony composition. The cultures should be kept in incubators that are opened as infrequently as possible. Opening incubators disturbs the oxygen and CO₂ levels inside the incubator, impairing the pH balance, and dramatically impairing the survival and proliferation of cells. When the cultures are refed, dump the old medium out of the plates and add fresh medium as quickly as possible and get them back into the incubator.

C. Low oxygen cultures

When NCSCs are cultured in nonadherent plates as neurospheres, they are grown in normal humidified tissue culture incubators with 6% CO₂ (and 20% oxygen). But when they are cultured in adherent cultures they are grown in low oxygen chambers to promote increased NCSC survival, proliferation, and improved differentiation. For low oxygen cultures, plates are inserted into gas-tight modular incubator chambers (Billups-Rothenberg, Del Mar, CA) that are flushed with a custom gas mixture containing 1% O₂/6% CO₂/balance N₂. The incubator chambers are flushed for one (sciatic nerve NCSCs) to one and a half (gut NCSCs) minutes daily at a rate of 15 liters per minute, then inserted into normal tissue culture incubators. This achieves an actual concentration inside the chamber of 3-6% oxygen, based on direct measurement with a microelectrode (Animus Corp., Malvern PA). Once cultures are established in the reduced oxygen chambers, opening of the gas-tight chambers is minimized in order to avoid reperfusion injury upon exposure to air. Nonetheless, these cultures are refed with new medium after 6 days according to the schedule described above, but their time outside of the low oxygen environment is minimized.

D. Immunohistochemical analysis of NCSC differentiation in culture

For routine analysis of culture compositions, cultures are fixed in acid ethanol (5% glacial acetic acid in 100% ethanol) for 20 minutes at -20°C, washed 3x in PBS, then blocked in PGN (PBS with 4% goat serum, 0.2% BSA and 0.1% NP-40) for 20 minutes, and stained with antibodies against peripherin (Chemicon AB1530, Temecula CA), smooth muscle actin (Sigma A-2547), and GFAP (Sigma G-3893) as described previously (Morrison et al., 1999). Primary and secondary antibodies are also dissolved in PGN. In order to stain for sympathetic markers, cultures are fixed in 4% paraformaldehyde for 10 minutes, blocked in PGN for 15 minutes, then stained with antibodies against tyrosine hydroxylase (Boehringer Mannheim, Indianapolis, IN), and dopamine-ß-hydroxylase (Pharmingen, San Diego CA), at room temperature for 2 hours. Better differentiation of adult NCSCs may also be achieved by allowing the cultures to continue in differentiation medium an extra day in culture.

6. Modifications for the culture of mouse gut NCSCs
Mouse NCSCs can most easily be cultured as neurospheres. These neurospheres can then be transferred to adherent culture for differentiation. Typically, cells should be cultured for at least 8 days before transfer to adherent cultures or analysis. We use low-binding plates (Clonetech) for the culture of neurospheres in non-adherent cultures, and PDL and Fn coated plates (as described above) for adherent cultures.

References that describe the culture of adult mouse gut NCSCs


Mouse self-renewal medium

To make 100 ml of medium:  
DMEM-low glucose, 50 ml  
Neurobasal, 30 ml  
Chick Embryo Extract (CEE) 15 ml  
Penn/Strep (P/S) 1 ml  
N2, 1 ml  
B27, 2 ml  
Retinoic Acid (RA; 117 \, \mu M \text{ stock } = 35\mu g/ml), 100 ul  
2-mercaptoethanol (2ME; 50 mM stock), 100 ul  
bFGF (25\mu g/ml stock), 80 ul  
IGF-1 (20 ng/ml of media)  
Fibronectin (0.83 ug/ml final or a 1:200 dilution of stock described above)