Self-renewal of Cultured Neural Crest Stem Cells (NCSCs)

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Subcloning Neural Crest Stem Cells in Culture

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The subcloning assay is used to assess the extent to which individual NCSCs self-renew in culture (the number of multipotent daughter cells generated per multipotent founder cell). Typically, single NCSCs are cultured 1 per well in 96 well plates for 6-8 days, then subcloned to fresh wells. The subcloned cells are cultured at clonal density (so that the potential of individual daughter cells from the original NCSC can be assessed) for two weeks exactly as primary NCSCs are cultured with 6 days standard medium followed by 8 days in medium that promotes differentiation

References that describe these methods for subcloning NCSCs include (see attached):


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

Set up plates by coating with poly-d-lysine and fibronectin and then adding standard NCSC medium as described in detail in the fetal NCSC isolation protocol for initial plating of NCSCs. For convenience, this information is repeated below.

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), 80 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
- Neural (N2), 1 ml
- B27, 2 ml
- Retinoic Acid (RA; 100 μM stock), 100 μl
- 2-mercaptoethanol (2ME; 50 mM stock), 100 μl
- bFGF (25μg/ml stock), 40 μl
- IGF-1 (20 ng/ml of media)

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.

C. 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).

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 pipette 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. Prepare Quenching Solution

The base is the same as the medium used for FACS sorting, but contains 2 mg/ml BSA and 10% CEE. Note that for standard medium, CEE that has only been frozen once is used. But for quenching solution CEE thawed and refrozen multiple times can be used.

Staining medium (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)

Add 15 ml CEE, per 150 ml staining medium to make final solution. Use 2 ml quenching solution per colony being subcloned out of a well of a 96 well plate in 80 ul trypsin. If larger numbers of cells are being subcloned (e.g. a well of a 6 well plate containing multiple colonies), dilute trypsin ~1:10 in quench. Typically prepare one centrifuge tube for
each sample containing the appropriate amount of quench solution before beginning to subclone the cells. These should be kept on ice until ready to use.

3. Replating the Cells

A. First examine wells to identify colonies to subclone. In the case of a 96-well plate where one cell is plated per well there may be many wells without colonies. NCSC colonies are typically large with densely packed small cells after 7-8 days of culture. Mark on the lid as you go, then mark the bottom of all the identified wells with a permanent marker to avoid subcloning the wrong wells once the lids are off.

B. Pre-warm 0.05% trypsin/EDTA solution (Gibco) at 37°C.

C. Dump off primary medium in into a sterile dish in the hood, removing as much as possible with a quick shake. Removing medium with a glass pipette and suction could result in all or part of the colony being removed. It is not necessary to rinse wells with PBS. Add 80 ul warm trypsin/well (96 plate) to each identified colony to subclone. For other sized plates, add enough trypsin to cover the bottom of the well. Place plate in incubator for 90 seconds.

D. Check under microscope to determine whether all cells have rounded and released from the bottom of the dish. If not, continue incubation as necessary at 37°C. If the trypsin solution is fresh, 2-3 minutes should be sufficient. Immediately transfer dissociated cells into pre-made centrifuge tubes containing quench solution. It is very important to minimize triturating the cells while they are in trypsin. Transfer all the trypsin and whatever cells come with it in one clean step into the quench solution, then pipette in the quench to disperse them. Then take a small amount of the quench to rinse the well and collect any remaining cells that may be stuck. Check under microscope at the end to make sure all the cells are being transferred.

E. Spin down cells at 1000 rpm (210 rcf) for 4 min at 4°C. Make sure the cells are ice cold before beginning centrifugation. Resuspend cells in 100-200 ul of staining medium. Plate either a set fraction of the total volume into wells of a 6 well plate (so that the total number of multipotent daughter cells per original colony can be calculated) or count cells by hemocytometer to plate a specific cell number and compute the total cells in the original colony. It is best to perform this step such that it will be possible to determine both the percentage of cells that formed secondary stem cell colonies, and the total number of secondary stem cell colonies per primary stem cell colony. Cells should be plated at clonal density. How many cells to plate will depend on how good cell survival is after your subcloning procedure, the age and type of NCSCs at isolation (embryonic NCSCs form larger colonies than adult) and the fraction of cells within each initial colony that will be capable of forming colonies themselves (this will vary with age and with amount of time in primary culture). Initially, it may be wise to try 2-3 wells/sample containing different numbers of cells such as 250, 500 and 1000 such that you don't end up with no colonies or only colonies that are too dense to identify individual clones. Ideally, you would like to have 40 large colonies/well. Roughly, you may estimate approximately 20% of the cells in E14.5 sciatic nerve NCSC colonies and 10% of the total cells in E14.5 gut NCSC colonies are capable of forming large multipotent colonies after 8 days in primary culture.

F. As with primary NCSCs, culture subclones in low oxygen chambers. 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, but their time outside of the low oxygen environment is minimized.

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-β-hydroxylase (Pharmingen, San Diego CA), at room temperature for 2 hours.

**Modifications of the Procedure for Subcloning of Mouse NCSCS**

Mouse NCSCs are most easily cultured as neurospheres. Individual neurospheres are often difficult to dissociate into single cells, therefore, they can first be transferred to adherent culture in 96 well plates for 1-2 days in mouse self-renewal medium and then subcloned as above for rat NCSCs. However, dissociated cells can be plated once again into ultra low binding plates for culture as neurospheres. Once neurospheres form, they should be transferred to adherent culture to assess multipotency of individual neurospheres.

**A. References that describe the culture of mouse gut NCSCs**


**B. Mouse self-renewal medium**
To make 100 ml of medium:  
DMEM-low glucose, 50 ml  
Neurobasal, 30 ml  
Chick Embryo Extract (CEE) 15 ml  
Pen/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), 80 ul  
IGF-1 (20 ng/ml of media)  
Fibronectin (0.83 ug/ml final or a 1:200 dilution of stock described above)