Scheel et al, 2005

Supplementary Protocol

Cell Culture

Rat Neural Stem Cells (NSCs)

A. Coating plates:

- Dissolve 100 mg of Poly-L-Ornathine (Sigma) in 10 ml of sterile water to make a 10 mg/ml stock solution. Store at -80°C.
- Laminin (Collaborative Biomedical Products) comes in solution. Aliquot ~1 ml solution into 4 tubes (250 µg/tube).
- Day 1: Dilute 1 aliquot of Poly-L-Ornathine into 50 ml water. Plate enough on dish to cover base. Incubate overnight at room temperature.
- Day 2: Wash plates twice with water, and then dilute 1 aliquot of laminin into 50 ml of sterile PBS. Incubate overnight in a 37°C incubator.
- Day 3: Can use plates immediately or store at -20°C for two months.

B. Media preparation:

- Stock media consists of 500 ml DMEM/HAMS F-12 1:1 (+high glucose w/o glutamine) (Omega Scientific) + 5 ml penicillin/streptomycin 10,000 µg/ml each (Cellgro) + 5 ml Fungizone 250 µg/ml (Omega Scientific) stored at 4°C.
- Working media consists of 50 ml Stock media + 0.5 ml N-2 supplement (GibcoBRL) + 0.5 ml L-glutamine 200 mM (GibcoBRL) stored at 4°C. Should be made fresh weekly.
- Culture media consists of 10 ml of Working media + 2 µl FGF-2 (final concentration 20 ng/ml) made fresh daily.
- Incubation media consists of 5 ml Working media + 5 ml CCg enriched media + 2 µl FGF-2 + 1 µl DNase.
- Sorting media consists of 5 ml Working media + 5 ml CCg enriched media + 2 µl FGF-2 + 1 µl DNase + 3 µl propidium iodide.
  i. Note: This represents the optimal conditions for maximum cell survival, however we have tried several concentrations and reagents (Supplemental Table 1b).
- Expansion media consists of 5 ml Working media + 5 ml CCg enriched media + 4 µl FGF-2

C. Culturing rat NSCs:

- Cells are grown to 60-80% confluency. Media should be changed every 3 days.
- When cells are at 80% confluency, they are split by discarding media with a vacuum pipet and adding 1 ml trypsin for 30-60 seconds. If preparing for single cell isolation by FACS, cells should be exposed for the least amount of time necessary to disassociate cells from the plate and each other. This is done by taping the side of the dish and checking for
Supplemental Table 1: Optimizing FACS for Isolation and Survival of Single Neural Stem Cells

<table>
<thead>
<tr>
<th>Method of cell detachment</th>
<th>Initial sample filter</th>
<th>Secondary sample filter (pressure)</th>
<th>Effect on single cell survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>B-D sample filter</td>
<td>70 µm (13.5 psi)</td>
<td>Normally used</td>
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<td></td>
<td></td>
<td>70 µm (10 psi)</td>
<td>none</td>
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<td></td>
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<td>200 µm (2 psi)</td>
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<td></td>
<td>B-D sample filter</td>
<td>70 µm (13.5 psi)</td>
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<td>70 µm (10 psi)</td>
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<td>200 µm (2 psi)</td>
<td>none</td>
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<tr>
<td></td>
<td>Nitex filter (75 µm mesh)</td>
<td>70 µm (13.5 psi)</td>
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<td></td>
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<td>70 µm (10 psi)</td>
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<td>Accutase</td>
<td>B-D sample filter</td>
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<tr>
<td></td>
<td></td>
<td>200 µm (2 psi)</td>
<td>none</td>
</tr>
</tbody>
</table>

b. Media | Effect on single cell survival
--- | ---
N2 | variable
CCg-enriched | variable
Mixture (1:2 or 1:4) | improved
DNase | improved
BSA | none
Propidium iodide | improved
c. CCF-2 concentration | Detected by FACS | Effect on single cell survival
--- | --- | ---
1X (0.75 µm) | 100% | no cells survive
1/2X (0.37 µm) | 100% | none
1/4X (0.19 µm) | 100% | small improvement
1/8X (0.09 µm) | 1/2 intensity | small improvement
1/16X (0.045 µm) | 1/4 intensity | small improvement
1X with wash | 100% | improved
without DMSO | 20% | none
d. Laser power on FACS | Effect on single cell survival
--- | ---
50 mW | improved
100 mW | normally used

B-D indicates Becton Dickinson, psi indicates pounds per square inch, CCg-enriched indicates conditioned media enriched with a glycosylated form of cystatin C, BSA indicates Bovine Serum Albumin, miliwatts indicates mW, red text indicates standard conditions.
detachment under the microscope. It is important not to over-trypsinize even if this translates into leaving some of the cells attached to the dish.

- 4 ml working media is added and mixed with the trypsinized cells and immediately spun down at 800 g for 2 minutes.
- If using cells for single cell isolation using FACS, see Using FACS for single NSC isolation and quantitative analysis below.
- Cells are resuspended in 1 ml Culture media using a beveled glass Pasteur pipet (use flame to bevel) and split 1:4.

D. Expansion of single cells into clones

- Media on single cells in a 96 well plate should not be changed for the first 9 days. Instead, add 50-100 µl of Expansion media every 3 days. Alternatively, supernatant from rat NSC cultures can be used with 2X and 3X FGF-2 on days 3 and 6, respectively.
- Cell survival should be assessed on day 3 post-FACS and cell expansion should be assessed on day 6.
- On day 9 post-FACS, the media should be changed to Culture media if the well is 20-30% confluent, otherwise fresh 1:1 Culturing media:CCg media with 2 µl FGF-2 should be added until the cells are 20-30% confluent.
- When the cells are 80% confluent, cells are split by removing the media and adding 40 µl trypsin for 10-20 seconds. 200 µl of Culturing media is added to the well and the cell suspension is transferred to a 24-well plate without spinning down cells.
- Split from a 24-well to a 6-well plate and then to a 10 cm dish.
- At this point, clones can be stored, analyzed for their trapped gene or used to further characterize the expression of the trapped gene (see Plate Reader, Using FACS for single NSC isolation and quantitative analysis, Microscopy sections below).

E. Differentiation of rat NSCs

- Astrocyte differentiation: NSCs are split into culturing media and grown overnight. The media is then changed to 10 ml Working media + 50 ng/ml leukemia-inhibitory factor (R & D Systems) + 50 ng/ml BMP2 (CHEMICON International, Inc.) + 0.1% BSA (Omega Scientific) for 3-7 days.
- Neuronal differentiation: NSCs are split into culturing media and grown overnight. The media is then changed to 10 ml Working media + 1 μM retinoic acid + 5 μM forskolin (Sigma-Aldrich) for 7 days.
- Oligodendrocyte differentiation: NSCs are washed in insulin-free media, then split into DMEM:F12 (1:1) with insulin-free N2 supplement and 500 ng/ml Insulin-like growth factor (R & D Systems) for 3-7 days.

Modifications made for culturing mouse progenitor cells

- Culturing dishes are not coated with laminin unless cells they are going to be differentiated.
Culturing media: 10 ml Working media + 2 µl FGF-2 (final concentration 20 ng/ml) + 2 µl EGF (final concentration 20 ng/ml) + 5 µl Heparin, made fresh daily.

Sorting media: 10 ml of the supernatant derived from a population of cultured mouse progenitor cells after 2 days of culture + 2 µl FGF-2 + 2 µl EGF + 5 µl Heparin + 1 µl DNase + 3 µl propidium iodide (no CCg is used).

Differentiation into neurons: NSCs are split into culturing media and grown overnight. The media is then changed to 10 ml Working media + 1 µM retinoic acid + 5 µM forskolin (Sigma-Aldrich) + 100 µl fetal bovine serum for 7 days.

i. Note 1: With these culturing conditions, one can expect a higher number of other differentiated cell types in the dish.

Otherwise culturing, CCF-2 loading and analysis, and single cell FACS isolation and expansion is the same.

i. Note 1: We have experienced a decrease in single cell survival and expansion following FACS. We believe that this is a result of the lack of specialized media, such as the CCg media for rat NSCs.

Using FACS for single NSC isolation and quantitative analysis

A. Cell preparation (continued from Culturing rat NSCs section):

- Prior to cell preparation, 50-75 µl of 1:1 Culturing media:CCg media with 2 µl FGF-2 should be added to each well in laminin-coated 96-well plates and incubated in a 37°C tissue culture incubator.
  i. Note 1: This allows CO₂ concentration and temperature to equilibrate to optimal cell growth conditions and increases consistency of cell survival between experiments.
  ii. Note 2: 200 µl of media is placed in the outer wells to prevent subtle changes in nutrient concentration (caused by evaporation) from influencing survival or behavior. No cells are sorted into these wells.

- Cells are resuspended in 1 ml of Incubation media using a beveled glass Pasteur pipet (use flame to bevel) and then add 5 µl CCF-2/DMSO (0.5X) to the cell suspension.

- Incubate cells at room temperature for 30-45 minutes. Cells should be placed in a rocker to prevent clumping and ensure good mixing with the substrate. Container should be wrapped in foil to minimize light exposure.

- Wash cells by adding 4 ml of Incubation media and centrifuging at 800 g, then resuspending in 1 ml of Sorting media (propidium iodide is added after cells are resuspended). Failure to wash cells will result in a decrease in cell survival (Supplemental Table 1c)
  i. Note: We have done experiments that suggest that the amount of CCF-2/DMSO can be decreased to ¼ the manufacturer’s instructions without a decrease in the dynamic range of the CCF-2, provided that the incubation time is kept < 1 hour (Supplemental Table 1c). We did not extend this study past 1 hour.
B. Sorting tagged cells

- Cells were sorted using a Becton Dickinson (San Jose, CA) FACS Star Plus equipped with a coherent INNOVA90 argon laser producing 50 mW of multiline ultraviolet excitation at 351-364 nm.
  i. Note 1: To minimize the exposure of the NSCs to UV light, the laser power was decreased from 100 mW to 50 mW (Supplemental Table 1d). There was no difference in survival between cells sorted at 2, 10, and 13.5 pounds per square inch (psi).
  ii. Note 2: The type of FACS machine can be substituted, provided that it is equipped with the appropriate UV laser and detection cubes.
  iii. Note 3: Although we have tested a variety of filter sizes on the cell sorter, as long as the size was greater than 70 µM, there was no effect on cell survival (Supplemental Table 1a).

- Fluorescence was detected via 450/20 (blue) and 560/30 (green) emission filters, separated by a 505 short-pass dichroic mirror.

- Prior to sorting the tagged cells, CCF-2 loaded nontransduced NSCs should be used to set-up gates to identify tagged cells with no β-lactamase expression (Green).
  i. Note 1: It is important to include this control with every experiment and analyze these cells by FACS before and after each experiment to control for any spontaneous hydrolysis of the substrate.
  ii. Note 2: A population of constitutively expressing β-lactamase NSCs, also loaded with CCF-2, should be used to set-up gates to identify tagged cells with β-lactamase expression (Blue) and to verify that enough substrate was present in the NSCs to saturate the β-lactamase enzyme during the entire experiment.
  iii. Note 3: Positive and negative control CCF-2-loaded NSCs should be visualized with a fluorescent microscope before and after each experiment to verify fluorescence.

- Tagged NSCs were sorted into subpopulations of expressing (blue) or not expressing (green) β-lactamase using the same technique as single “Blue” and “Green” tagged cells.

C. Using FACS to calculate the blue/green ratio of tagged clones

- Established tagged clones can be maintained in culturing media, however NSCs should not be split beyond passage 30 for analysis of gene expression in response to a biological stimulus.

- The loading of the NSCs with CCF-2 and gates on the FACS should be set up as described above; however in the experiments involving differentiation of NSCs, the controls should include differentiated nontransduced NSCs because the size and shape of the cells will vary depending on the differentiated cell type.
For each experiment, 10,000 cells that were included by each gate were analyzed by FACS for their average blue and green fluorescent intensities. This number of cells can easily be obtained from a well in a 6-well plate.

Given the diversity of cells that develop in a population of NSCs, we analyzed 5 separate experiments for each tagged clone at each time point, however this did not change the standard error. We recommend that each experiment be done in triplicate.

To eliminate potential differences in background fluorescence between different conditions, each blue/green ratio of the tagged clones is corrected using a correction factor derived from the blue/green ratios of the nontransduced control cells cultured in the same conditions.

To calculate the corrected blue/green ratios for each tagged clone, their blue/green ratios were multiplied by a correction factor, derived from the division of the blue/green ratio from the undifferentiated nontransduced cells by the blue/green ratio of the differentiated nontransduced cells.

Using a plate reader to detect CCF-2 cleavage by β-lactamase

For each stimulus tested, load three wells with 20,000-40,000 cells/well in a 96-well plate. The amount of cells loaded will depend on how long the cells will be cultured prior to analysis and what condition is being tested, but optimal detection of fluorescence occurs at cell densities >40,000 cells/well. For example, differentiated cells will not divide and therefore wells can be seeded with a higher number of cells.

When ready to measure the β-lactamase expression of adherent NSCs, the media is replaced with fresh media + 3 µl CCF-2/DMSO for every 10 ml of media. The type of media will depend on the stimulus being measured.

Cells are incubated at room temperature for 30-45 minutes and kept stationary, wrapped in foil.

i. Note 1: NSCs incubated in CCF-2/DMSO tend to migrate towards the center. This is important to remember when selecting where the plate reader scans the cells in the well.

ii. Note 2: If blue and green fluorescence of one well differs from the other two wells, then the wells should be visualized under the microscope to verify that the cells have not floated off the bottom of the dish. Make sure not to overexpose the cells to the UV light (this artificially increases the blue fluorescence).

The same control cells used for FACS analysis should be included in each plate being used.

A Cytofluor 4000 plate reader (Applied Biosystems, CA) was used for all experiments. CCF-2 was detected with an excitatory 409/20nm UV light and emission was detected with a 460/40nm (cleaved CCF-2) and a 530/25nm (uncleaved CCF-2) band pass filters.

i. Note 1: We have used several different types of plate readers with this system and all work, however for optimal results, the cubes
should be within the specifications of the β-lactamase/CCF-2 system described by Aurora/Panvera (San Diego, CA).

- Use the blue and green fluorescent intensities, measured by the plate reader, to calculate the blue/green ratio for each well.
- Calculate the corrected blue/green ratios for each well by multiplying each ratio by a correction factor derived from the division of the blue/green ratio from the unexposed nontransduced cells by the blue/green ratio of the exposed nontransduced cells.

Using a microscope to detect CCF-2 cleavage by β-lactamase

- Cells are split into 96-well plates and loaded with CCF-2 media as described above. Again, the type of media and cell density will depend on the conditions being tested.
- Visualize cells and acquire images on a microscope (We used a Model TE2000; Nikon) equipped with a 20X fluorescence objective lens, an excitatory 405/20 nm UV light, an emission 435 nm long pass filter, and a 425 nm dichroic mirror.
- Images can be post-processed using Adobe Photoshop®, however this system involves the use of a ratiometric fluorophore, therefore any adjustments in fluorescent intensity should be done simultaneously in both the blue and green channels.