Single Cell Seeding of BBB Stem Cell Model

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ABSTRACT
Standardized single cell seeding protocol for Blood-Brain Barrier (BBB) differentiation.

ATTACHMENTS
Standardized_single_cell_seeding_protocol_for_BBB_differentiation_(Lippmann_Lab_updates).pdf

MATERIALS

<table>
<thead>
<tr>
<th>NAME</th>
<th>CATALOG #</th>
<th>VENDOR</th>
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</thead>
<tbody>
<tr>
<td>UltraPure™ DNase/RNase-Free Distilled Water</td>
<td>10977023</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>Gibco™ DPBS no calcium no magnesium</td>
<td>14190144</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>StemPro™ Accutase™ Cell Dissociation Reagent</td>
<td>A1110501</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>Countess™ II Automated Cell Counter</td>
<td>AMQAX1000</td>
<td>Thermo Fisher</td>
</tr>
<tr>
<td>Y-27632 dihydrochloride (Rock Inhibitor)</td>
<td>1254/10</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>Countess™ Cell Counting Chamber Slides</td>
<td>C10312</td>
<td>Thermo Fisher Scientific</td>
</tr>
</tbody>
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MATERIALS TEXT
- Corning tissue culture plates
- 15 ml conical tubes
- Microfuge tubes
- EB8 media prepared in-house

SAFETY WARNINGS
Please see SDS (Safety Data Sheet) for hazards and safety warnings.

BEFORE STARTING
- Matrigel Plates should be ready to use at start of procedure.
- Procedure was optimized using IMR90-4 pluripotent stem cells. The procedure has successfully been extended to CC3, CD12, SM14, and DSP-mEGFP pluripotent stem cells.

Reagent Preparation
1. ROCK inhibitor:
   - Make 10 Millimolar (mM) working stock solution by diluting 10 mg ROCK inhibitor into 3.12 ml ultrapure distilled water. Use at 1:1000 for 10 Micromolar (µM) final concentration. Aliquots can be stored long term at -80 °C for up to 1 year and frozen/thawed as many times as necessary.
   - EB8 media

Seeding cells for BBB differentiation using single cell seeding (Day -1)
2. Manually transfer spent medium to a 15 ml conical. Save 1 ml of media for every well being passaged.

Citation: Ethan Lippmann, Hannah Wilson, Emma Neal (03/24/2020). Single Cell Seeding of BBB Stem Cell Model. https://dx.doi.org/10.17504/protocols.io.8j9hur6
3 Wash each well once with 2 ml PBS.

4 Add 1 ml accutase (warmed to Room temperature) to each well.

5 Incubate at 37 °C until the cells are beginning to detach (approx. 00:03:00 – 00:05:00).

6 Using p1000, collect cells, and spray gently over surface 2 – 3x to dislodge any remaining cells. Pipetting more than this will reduce cell viability.

7 Collect cells in the 15 ml conical containing spent medium.

8 Spin down cells for 00:04:00 – 00:05:00 at 1000 rpm.

9 Aspirate media, resuspend cells in 1 ml E8 medium. Thoroughly tritrate 2 – 3 times using p1000 to yield single cell suspension.

10 Take 10 µl of cells to count, drawing from the middle of the sample to prevent bias from settling cells. Transfer these cells to a clean microfuge tube.

11 Dilute the 10 µl of cells in 10 µl of 0.4 Mass Percent Trypan blue. Transfer 10 µl of this diluted suspension to a Countess cell counting chamber; allow to sit for 00:00:30.

   Note that this step is performed outside the hood and is NONSTERILE.

12 Insert the slide into the Countess II automated cell counter. Note the calculated live cell density. You will use this density (not the total density) to calculate the number of cells needed for seeding.

13 Calculate appropriate volume of cells to add to each 6-well.
   - Typical seeding number for IMR90-4 iPSCs is between 100,000 – 120,000 cells per well.
   - Typical seeding number for CC3 and CD12 iPSCs is 150,000 cells per well.

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14 Resuspend cells in appropriate volume of E8 + [10 Micromolar (µM) ROCK inhibitor (2 ml/well)]

If you have excess cells, discard the excess before resuspending.

15 Place plate in incubator, and shake plate quickly back and forth (not swirling) to distribute cells evenly.

16 Approximately 24 hours later (Day 0), initiate differentiation:

16.1 Note: Cells are seeded for differentiation in E8 medium according to the standardized single cell seeding protocol

On day 0, aspirate E8 medium and add 2 ml of E6 per well.

16.2 Change medium every day using 2 ml of E6 per well.

16.3 At day 4 of E6 treatment, aspirate and add 2 ml of EC medium with bFGF (basic fibroblast growth factor) and [10 Micromolar (µM) RA] to each well.

Medium is NOT changed during expansion phase.

16.4 BBB subculturing:
On day 6, subculture BBB onto plates and Transwell filters according to the following protocol:

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