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# 2D differentiation of NPCs to neurons & astrocytes

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**Protocol status:** In development
**We are still developing and optimizing this protocol**

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## Abstract

Protocol to differentiate neural progenitor cells to mature neurons and astrocytes in 2D culture.

## Protocol materials

Corning® Matrigel® Basement Membrane Matrix, LDEV-free, 10 mL **Corning Catalog #**354234

Corning® Matrigel® Basement Membrane Matrix, Phenol Red-free, LDEV-free, 10 mL **Corning Catalog #**356237

## Overview

1 Typical workflow:

d(-14) thaw the NPCs (in ROCKi)
- maintain in +EGF+FGF

d(-7) passage (in ROCKi)
- maintain in +EGF+FGF

d0 passage (in ROCKi)

d1 full media change to start differentiation to neurons/astrocytes

d7 passage (without ROCKi)

d14 passage (without ROCKi)

d42 Mitomycin C treatment (**for neurons only**)

~d60-100 replate neurons/astrocytes for experiments

> Note
>
> If more cells are needed, the NPCs can be maintained additional passages in +EGF+FGF before differentiation, but can't be refrozen and thawed again. If frozen/thawed again, they don't proliferate or differentiate well.

## Media Recipe

2 **NPC Base Media (500mL):**

| | |
|---|---|
| 240mL | 50% DMEM/F12 (Hyclone VWR Cat # SH30023.02) |
| 240mL | 50% Neurobasal (FisherSci Cat # 21103-049) |
| 2.5mL | 0.5X N2 (100X; Invitrogen Cat # 17502048) |
| 5mL | 0.5X B27 without VA (50X; Invitrogen, 12587010) |
| 5mL | 1X Glutamax (100x Invitrogen Cat # 35050061) |
| 5mL | 1X NEAA (Invitrogen Cat # 11140050) |
| *250 uL | 5 ug/mL Human Insulin Solution (250mg per 500mL) (Sigma Cat # I9278-5ML) |
| 1mL | 1X Primocin (stock 500X, InvivoGen ant-pm-2) |
| 50uL | 1 µg/mL Heparin (10 mg/ml stock Sigma H3419-100KU) |

Note

*Look up the lot number of human insulin & make aliquots of 2.5mg.
The concentration is typically 9-11 mg/mL -> 2.5 mg will be ~250uL.

**For NPC maintenance:**
+20 ng/mL bFGF (stock 100 ug/mL): 10uL/50mL NPC base
+20 ng/mL EGF (stock 200 ug/mL): 5uL/50mL NPC base

**For Neuron differentiation:**
d0-d80+
+20 ng/mL BDNF (stock 200 ug/mL): 5uL/50mL NPC base
+20 ng/mL GDNF (stock 100 ug/mL): 10uL/50mL NPC base
+1 uM cAMP (stock 10mM): 5uL/50mL NPC base

~d42 (can be d42-d49)
1hr pulse with 5 ug/mL Mitomycin C ready made solution (stock 10 mg/mL Sigma Cat# M5353)
^^ this is enrich for neurons, dividing cells die off or stop proliferating over the next 4-7 days

**For Astrocyte differentiation:**
d0-d14
+10 ng/mL hLIF (stock 100 ug/mL): 5uL/50mL
+10 ng/mL EGF (stock 200 ug/mL): 2.5uL/50mL

d14-d80+
+10 ng/mL hLIF (stock 100 ug/mL): 5uL/50mL
+10 ng/mL CNTF (stock 100 ug/mL): 5uL/50mL

## Thawing Neural Progenitor Cells (NPCs)

3 See protocol for thawing hematopoietic progenitor cells (HPCs).
https://www.protocols.io/view/thawing-frozen-hematopoietic-stem-cells-hpcs-dddt226n

**It is the same procedure for NPCs, except:**
- the plates are coated with **0.5 mg/mL standard matrigel**
- the cells are maintained in **NPC base +EGF+FGF+10uM ROCKi** (ROCKi is removed 24hr after thawing)

- the cells are plated at **250-300K/well in a 6w plate**

## Passaging NPCs (every 6-7 days - volumes listed at for 1w of a 6w plate)

4 Before starting, coat 6-well plates with 0.5 mg/mL standard matrigel (Set aside for later) 30m

Note

**Use standard matrigel (regular or phenol-free):**

Corning® Matrigel® Basement Membrane Matrix, LDEV-free, 10 mL **Corning Catalog #**354234

Corning® Matrigel® Basement Membrane Matrix, Phenol Red-free, LDEV-free, 10 mL **Corning Catalog #**356237

**To make aliquots of matrigel:**

- The concentration of a lot of matrigel can be looked up here in "quality certificate lookup" section. (https://www.corning.com/worldwide/en/products/life-sciences/resource-library.html).
- To keep coating consistent across lots, I check the concentration and make aliquots. However, if you don't have the lot number information, 0.5mg/mL is close to a 1:20 dilution.

**To coat plates:**

- Thaw an aliquot of matrigel at 4 °C or On ice
- Dilute with the appropriate amount of ice cold DMEM/F12
  6.5 mL for 6.5-wells = 3.25 mg matrigel
- Aliquot 1 mL per well in a cold 6-well plate
- Shake/tilt the plate until the matrigel evenly coats the bottom
- Incubate 37 °C for 00:30:00
- After incubating, aspirate the matrigel
- Add 2 mL per well DMEM/F12
- Place the plate in the incubator at 37 °C

Note

Plates can be stored in the incubator with DMEM/F12 for up to 1 week before use

4.1 Pre-treat the cells with [M] 10 micromolar (µM) ROCK inhibitor (ROCKi) 1h 30m

- Pick up the media in the well to a 15mL conical tube.
- Add [M] 10 micromolar (µM) ROCKi ( 2 µL per well ) and mix well.
- Gently return the media to the dish.
- Incubate the cells with ROCKi for 00:30:00 - 01:00:00 at 37 °C

4.2 Lift the cells with Accutase.

- Aspirate the media.
- Wash with 2mL/well PBS (without Ca/Mg).
- Aspirate the PBS.
- Add 1mL/well Accutase.
- Incubate 5 minutes at 37C.
- Pick up the cells in accutase with a glass pipette and blow the cells off the dish.
- Transfer the cells to a 15mL conical tube.
- Add 6mL NPC base media to the dish and blow the remaining cells off the dish.
- Transfer the remaining cells to the 15mL conical tube.
- Pipette up/down 3-5x in the conical tube to break apart clumps. To break apart clumps, pipette the cells up, and then press the pipette against the side of the tube and blow out quickly.
- Pick up the entire cell mixture and filter through a 100um cell strainer over a 50mL conical tube to remove any remaining large clumps.
- Transfer the cell mixture back to the 15mL conical tube.
- Centrifuge at 180g for 5 minutes room temperature.
- Aspirate the media.
- Close the conical tube lid and tap the pellet to loosen the cell pellet.
- Resuspend in 2-3mL of NPC base media.
- Pipette up/down 3-5x or until the cell solution is cloudy.

4.3 Count the cells and aliquot to new plates.

- Count 10uL cells + 10uL trypan blue with a hemocytometer.
- Determine the #uL for 1 million cells.
- Add 1 million cells/well in 2 mL per well NPC+EGF+FGF+ROCKi in new 0.5 mg/mL matrigel coated plates.

  **NPC+EGF+FGF+ROCKI (10mL):**

  10mL NPC base media
  1uL 20ng/mL EGF (stock 200ug/mL)
  2uL 20ng/mL FGF (stock 100ug/mL)
  10uL 10uM ROCKi (stock 10mM)
- Gently place the plate in the incubator and shake evenly (4x forward/back, 4x side-to-side, 4x forward/back).

Note

If the cells were confluent, 1 million cells/well is typically around a 1:2-1:4 passage dilution.

## Expansion of NPCs

5 d1 after passaging NPCs (see STEP 3)

- Perform a full media change and replace with NPC maintenance media without ROCKi (NPC base +EGF+FGF)

  **NPC+EGF+FGF (10mL):**

  10mL NPC base media

  1uL 20ng/mL EGF (stock 200ug/mL)

  2uL 20ng/mL FGF (stock 100ug/mL)

d3

- Perform a half media change. Remove 900uL/well, and add a fresh 1mL/well NPC base +EGF+FGF.

d5

- Perform a half media change. Remove 900uL/well, and add a fresh 1mL/well NPC base +EGF+FGF.

d6-7

- Passage to new dishes coated with 0.5mg/mL standard matrigel in NPC base +EGF+FGF.

  for 6w plates → 1 million cells/well in 2mL NPC base +EGF+FGF

  for 5cm cellBIND dishes → 2.4 million cells/dish in 6mL NPC base +EGF+FGF

Note

The high plating density is important, the cells need to be very dense to maintain progenitor identity.

I've stains the cells after maintaining in +EGF+FGF at high density up to 5x passages after thawing and > 90% of the cells remain PAX6+SOX2+NESTIN+. If passaged at lower density, the cells will stop dividing and/or differentiate.

## Differentiation of NPCs to Neurons

6 d1 after passaging NPCs (see STEP 3)

- Perform a full media change and replace with neuron differentiation media (NPC base +BDNF+GDNF+cAMP)

**NPC+BDNF+GDNF+cAMP (10mL):**

10mL NPC base media
1uL 20 ng/mL BDNF (stock 200 ug/mL)
2uL 20 ng/mL GDNF (stock 100 ug/mL)
1uL 1 uM cAMP (stock 10mM)

d3

- Perform a half media change. Remove 900uL/well, add a fresh 1mL/well NPC base +BDNF+GDNF+cAMP.

d5

- Perform a half media change. Remove 900uL/well, add a fresh 1mL/well NPC base +BDNF+GDNF+cAMP.

d7 [*differentiation passage 1 - no ROCKi during this step*]

- Passage to new dishes coated with 0.5mg/mL standard matrigel in NPC base +BDNF+GDNF+cAMP.
  for 6w plates → 1 million cells/well in 2mL media
  for 5cm cellBIND → 2.4 million cells/dish in 6mL media
  for 10cm cellBIND dishes → 5.8 million cells/dish in 12mL media

d9

- Perform a full media change and replace with neuron differentiation media (NPC base +BDNF+GDNF+cAMP) to remove any dead cells.

d11

- Perform a half media change. Remove 900uL/well, add a fresh 1mL/well NPC base +BDNF+GDNF+cAMP.

d13 [*differentiation passage 2 - no ROCKi during this step*]

- Passage to new dishes coated with 0.5mg/mL standard matrigel in NPC base +BDNF+GDNF+cAMP.
  for 6w plates → 400K cells/well in 2mL media
  for 5cm cellBIND → 1 million cells/dish in 6mL media
  for 10cm cellBIND dishes → 2.5 million cells/dish in 12mL media

d16+

- Perform a half media change every 3-4 days until 90-95% confluent. When the cells are 90-95% confluent, passage at the same density as d13 and continue with half media changes every 3-4 days until 90-95% confluent again.

for 5cm cellBIND → remove ~2-2.5mL (depending how much media evaporated, leave enough media
to cover the bottom), and add a fresh 3mL/well NPC base +BDNF+GDNF+cAMP.
for 10cm cellBIND → remove ~4.5-5mL (depending how much media evaporated, leave enough media
to cover the bottom), and add a fresh 6mL/well NPC base +BDNF+GDNF+cAMP.

Note

Seeding at the lower density and feeding the cells less often (every 4 days) results in more progenitor differentiation, plan to have around the number of dishes you want to end with after the 2nd differentiation passage.

~d42

- To enrich for neurons, around d42 (or close to this date when the dishes are 95% confluent again), treat the cells with 5ug/mL Mitomycin C for 1hour at 37C.
- After 1hr, perform 3x washes with 6mL PBS, and then replace with 6mL (5cm dishes) or 12mL (10cm dish) of fresh NPC base +BDNF+GDNF+cAMP.

Note

If the cells were ~95% confluent before Mitomycin C treatment, 1x 10cm typically yields around 3-6 million cells.

Continue performing half media changes until used for experiments. After Mitomycin C treatment, the cells should not expand further, so they likely won't need to be passaged again until plating for experiments.

*I've been using these for experiments ~d60-100.*

## Differentiation of NPCs to Astrocytes

7 d1 after passaging NPCs (see STEP 3)

- Perform a full media change and replace with astrocyte differentiation media #1 (NPC base +hLIF+EGF)

NPC+hLIF+EGF (10mL):
10mL NPC base media
1uL 10 ng/mL hLIF (stock 100 ug/mL)
0.5uL 10 ng/mL EGF (stock 200 ug/mL)

d3

- Perform a half media change. Remove 900uL/well, and add a fresh 1mL/well NPC base +hLIF+EGF.

d5

- Perform a half media change. Remove 900uL/well, and add a fresh 1mL/well NPC base +hLIF+EGF.

d6-7 [*differentiation passage 1 - no ROCKi during this step*]

- Passage to new dishes coated with 0.5mg/mL standard matrigel in NPC base +hLIF+EGF.
  for 6w plates → 1 million cells/well in 2mL media
  for 5cm cellBIND → 2.4 million cells/dish in 6mL media
  for 10cm cellBIND dishes → 5.8 million cells/dish in 12mL media

~d9

- Perform a full media change and replace with neuron differentiation media (NPC base +hLIF+EGF) to remove any dead cells.

~d11

- Perform a half media change. Remove 900uL/well, and add a fresh 1mL/well NPC base +hLIF+EGF.

~d13-14 [*differentiation passage 2 - no ROCKi during this step*]

- Passage to new dishes coated with 0.5mg/mL standard matrigel in **NPC base +hLIF+CNTF.**
  for 6w plates → 400K cells/well in 2mL media
  for 5cm cellBIND → 1 million cells/dish in 6mL media
  for 10cm cellBIND dishes → 2.5 million cells/dish in 12mL media
  **NPC+hLIF+CNTF (10mL):**
  10mL NPC base media
  1uL 10 ng/mL hLIF (stock 100 ug/mL)
  1uL 10 ng/mL CNTF (stock 100 ug/mL)

~d16+

- Perform a half media change every 3-4 days with **NPC base +hLIF+CNTF** until 95-100% confluent.
- When the cells are 95-100% confluent, passage at the same density as ~d13 and continue with half media changes with **NPC base +hLIF+CNTF** every 3-4 days until 95-100% confluent again.
  for 5cm cellBIND → remove ~2-2.5mL (depending how much media evaporated, leave enough media
  to cover the bottom), and add a fresh 3mL/well **NPC base +hLIF+CNTF**.

for 10cm cellBIND → remove ~4.5-5mL (depending how much media evaporated, leave enough media
to cover the bottom), and add a fresh 6mL/well **NPC base +hLIF+CNTF**.

Note

If the cells were ~95% confluent before passaging, 1x 10cm typically yields around 9-14 million cells.

The cells will expand slower with each passage, avoid passaging until 95-100% confluent. Continue performing half media changes until used for experiments, around d50-60 astrocytes with more complex branching should appear and increase in number.

*I've been using these for experiments ~d60-100.*

*After replating for experiments or if maintained past d100, I change the media to NPC +BDNF+GDNF+cAMP to prevent the astrocytes from overgrowing and detaching from the plate.*