How To: Differentiate pluripotent stem cells to cortical or hypothalamic neurons

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To date, most of our understanding of human biology has been extrapolated from rodents. While these systems are a valuable resource for understanding *in vivo* biology, there are key differences between humans and rodents, and models that enable us to understand human-specific aspects of molecular and cellular biology as well as disease have been lacking. Over the last decade, researchers have developed methods using human pluripotent stem cells (hPSCs) to generate specific cells types, providing new model systems for probing human biology and disease, as well as delivering novel drug screening platforms. Pluripotent stem cells have the capacity to differentiate into any cell type. By extrapolating what has been learned from developmental biology, a host of specialized cell types can now be generated in culture, including germ cells, cardiac cells, endothelial cells and neural cells.

The human brain contains billions of neurons, encompassing a large number of cellular subtypes which generate complex circuits with a myriad of specialized functions. The cerebral cortex comprises the largest part of the brain, responsible for a host of critical functions such as cognition, perception and decision making. It integrates signals from other parts of the brain, such as the hypothalamus, an area responsible for regulating key homeostatic processes such as feeding, sleeping and body temperature.

Both the cortex and hypothalamus are located in the forebrain, with the cortex located dorsally, while the hypothalamus is ventral. Thus, the key to generating these brain areas using hPSCs in vitro is to manipulate the cellular pathways that are critical for the specification of dorsal and ventral forebrain. The aim of this guide is to outline protocols for the differentiation of hPSCs to either cortical neurons or hypothalamic neurons, and to provide key pointers for researchers who are undertaking these protocols for the first time. For further reading and more in-depth protocols, we recommend you see: (Kirwan et al., 2017; Merkle et al., 2015; Shi et al., 2012b, 2012a).

1 Maintenance of pluripotent stem cells: The key to everything

The most important parameter in the efficient generation of any cell type from hPSCs is the proper maintenance of your hPSC cultures. While maintaining hPSCs on mouse embryonic fibroblasts (feeders) is the gold standard, this method is technically cumbersome. We therefore culture our cells in feeder-free conditions, on a substrate of Geltrex in mTeSR medium. When thawing your hPSCs, make sure they are at a sufficient density, generally 20,000-30,000 cells per cm^2, and culture overnight in ROCK inhibitor to prevent excessive cell death.

Do not keep the cells in ROCK inhibitor for more than 24 hours at a time, as this can create a selection event that can cause cell lines to become dependent on ROCK inhibitor.

Blocking cell death can also select for unwanted mutations in oncogenes such as p53. These mutations can also accumulate when cells are subjected to stresses or population bottlenecks caused by excessive or overly rigorous passaging, poor incubator maintenance (not maintaining the humidity or incorrectly calibrated temperature and CO_2), or not adhering to a strict media changing routine.

The media must be replaced every day, making sure that the media has reached room temperature before changing.

Do not warm the media in the water bath, as FGF2, a cytokine present in mTeSR and critical for hPSC proliferation, degrades quickly at 37˚C.

Allow hPSCs to expand as colonies, keeping an eye on the cellular morphology. Undifferentiated hPSCs are large (10-30 µm), with a high nuclear to cytoplasmic ratio. Passage the hPSCs when the colonies become close to merging or are approximately 80% confluent. Do not allow hPSCs to become over-confluent as they will start to spontaneously differentiate.

Passage the cells using 1mM EDTA. When passaging, do not keep the cells in EDTA for more than 5 minutes. Make sure you pipette gently when detaching the cells, as rigorous pipetting can result in excessive cell death. If the cells do not detach easily, incubate in EDTA for a few more minutes, monitoring the colonies constantly for detachment. Passage at a ratio of 1:4-1:10 depending on your starting density, or at 20,000-30,000 cells per cm^2 if using a cell counter.

Make sure you keep adequate frozen stocks of your hPSCs, at a low passage number (< 30 passages). Also note that you should not overly depend on certain hPSC lines. Some lines are easier to work with, and make neurons more efficiently, so it is worth experimenting with a few lines and finding the ones that work best for you. Keep an eye out for sudden changes in growth kinetics in you hPSC lines, as this may be an indication of unwanted mutations or chromosomal abnormalities.

2 Preparing cells for neural differentiation

Expand your hPSCs to so that you have sufficient numbers of cells to carry out a differentiation. We typically use a 10 cm dish of hPSCs for a neural differentiation. If you want to grow fewer cells, you can use a six-well plate. When the cells reach 80% confluence, and look like they need a passage, they can be detached and plated for differentiation. We detach and dissociate into a single cell suspension using TryPLE, however other enzymes such as Accutase also work well. When cells have been detached, washed, and resuspended in mTeSR media (containing Rock inhibitor), count them, and plate at a density of 100,000 cell per cm^2 onto dishes coated with Geltrex or Matrigel. The following day, the cells should be at 70-80% confluence and ready to start differentiation. If they haven't reached this density, change the media (mTeSR containing Rock inhibitor) and leave the cells an additional day to acquire confluency.

Do not start the differentiation if they are not sufficiently dense as you will achieve poor efficiency.

3 Cortical differentiation

When the cells are ready to start the differentiation, wash the cells with PBS, and add cortical differentiation media: N2B27 media containing the TGF inhibitors SB431542 (10 µm) and LDN193189 (100 nM) and the WNT inhibitor XAV939 (2 µm). Use 20 mLs for a 10 cm dish or 4 mLs for a six-well plate. Treat for 12-days with cortical differentiation media, changing the media every 2-days.

Monitor the cells and note that they should reach confluence by day 2. By day 4 they should become rounded up and uniform in appearance, with small clear nuclei. Between day 4-12 the cells should become further compacted, without any discernible nuclei and may eventually contain some

neural-ridge-like folds, where the cells clump to form narrow elongated elevated structures. You may notice other patchworks of irregular shaped cellular compactions. Together, these changes in cell morphology and appearance of the monolayer are indicative of efficient neuroectoderm formation. On day 12, cells should be dissociated into single cells using TryPLE, and passaged 1:3 onto Geltrex coated dishes, so that cells are still 100% confluent after passaging.

It is important to keep the confluence high to discourage the formation of contaminant neural crest-derivatives which can rapidly outgrow neuronal progenitors.

Between day 12-16, culture the cells in N2B27 20 mg/mL FGF2 to aid with proliferation, and in just N2B27 from D16 onwards. Around day 16 you should notice the formation of neural rosettes, polarised circular structures containing approximately 30-100 neural progenitor cells.

If cells have not formed rosette structures by day 20, it is likely that the differentiation was not successful, and it is advised that you start the differentiation again.

Passage at a ratio of 1:3 the cells again on day 20, using a TryPLE/Papain mixture, again keeping the cells at high confluence to avoid contamination with neural crest. From day 20, you will notice the formation of neurons, often at the edges of the rosettes. Carry out the terminal plating between day 30-35 using TryPLE/Papain into the desired format depending on the experiment that you wish to carry out. Cultures can be maintained up to and past 100 days.

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Hypothalamic differentiation

As for the generation of cortical neurons, prepare the cells as in section 2 and begin the hypothalamic differentiation when cells are greater than 70% confluence. Hypothalamic differentiation is carried out by first inducing a forebrain neuroectodermal fate as with cortical differentiation, but then exposing the cells to ventralising Sonic Hedegehog signalling agonists. Finally, neurogenesis is induced by inhibition of NOTCH signalling. Keep an eye out for the key morphology changes that occur during neuroectoderm formation (as described in the cortical differentiation). The key difference is that hypothalamic progenitors do not form neural rosettes, and their progenitors are larger and flatter in their morphology.

Differentiation is carried out in N2B27 media with the addition of small molecules as follows:

- Day 0: SB431542 (10 µm), LDN193189 (100 nM), XAV939 (2 µm)
- Day 2: SB431542 (10 µm), LDN193189 (100 nM), XAV939 (2 µm), SAG (1 µm), Purmorphamine (1 µm)
- Day 4: SB431542 (7.5 µm), LDN193189 (75 nM), XAV939 (1.5 µm), SAG (1 µm), Purmorphamine (1 µm)
- Day 6: SB431542 (5 µm), LDN193189 (50 nM), XAV939 (1 µm), SAG (1 µm), Purmorphamine (1 µm)
- Day 8: SB431542 (2.5 µm), LDN193189 (25 nM), XAV939 (0.5 µm), DAPT (5 µm)
- Day 10: DAPT (5 µm)
- Day 12: DAPT (5 µm)
- On day 14, dissociate the cells using TryPLE and replate onto Geltrex coated dishes at a density of 200,000 cells per cm^2.

Change the media the following day to N2B27 with 10ng/mL BDNF and change the media every other day.

Plan out the experiments that you wish to carry out with the cells and re-plate the cells into your desired format (e.g. 24-well plate) at any point up to day 35. We recommend detaching the cells using TryPLE/Papain and plating onto Geltrex coated dishes at a density of 200,000 cells per cm^2 for most experiments, but lower densities for imaging e.g. 100,000 cells per cm^2.

5 Quality control

When re-plating on D14, plate some cells into 3 wells of a 24-well plate for immunocytochemical staining. On day 15, fix some of the cells in 4% Formaldehyde solution and carry out a staining for transcription factors expressed in the dorsal forebrain (cortex) or ventral forebrain (hypothalamus). Cortical progenitors should express FOXG1, OTX1/2, PAX6 and should be predominantly organised into rosette structures. Most hypothalamic progenitors should express NKX2.1, in addition to some RAX, OTP and SIM1 expression.

Fix the remaining cells on day 25-30 and stain for neuronal proteins. Cortical neurons should express TBR1 and CTIP2, as well as the pan-neuronal markers MAP2 and 3-Tubulin. Older cortical neurons (day 50-100) should express late born cortical transcription factors SATB2 and CUX1. Hypothalamic cultures should contain POMC expressing cells (approx. 5-10% of cells) as well as pan-neuronal markers MAP2 and 3-Tubulin. Older hypothalamic cultures should express NPY, AGRP and HCRT.

For details of antibodies and staining protocols, see (Kirwan et al., 2017; Merkle et al.).

6 Neural culture maintenance

Cells can be maintained past 100 days of culture in N2B27 with 10 ng/mL BDNF. Change the media every other day. Monitor the colour of the media, making sure it does not become too acidic (orange-yellow). Increase the media volumes if this is the case to maintain a pink colour (neutral pH). Take care when changing the media, make sure the media is at room temperature or above, leave a small amount of media in the wells to prevent drying out and do not pipette too vigorously.

After day 50, the cultures become prone to detaching. Avoid plating at very high densities (above 200,000 cells per cm²) to prevent this.

If cells appear to be clumping or detaching from the edges of the wells, add 10 µg/mL laminin on a weekly basis to promote adherence to the plastic. We recommend growing cells on plastic as adherence is better in comparison to glass. If you wish to perform imaging, optical grade plastic dishes are available from Ibidi and other suppliers. We avoid growing the cells for prolonged periods on glass as they attach poorly.

References

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