Making iPS aggregates/"Embryoid bodies" (Day 0-3)
From iPSCs on MEFs

Reagents needed:

- iPSC media
- Scraper
- 15mL conical tubes
- PBS
- T-25 or T-75 (non-cell culture treated – white caps)

1. Scratch off "bad" iPSC colonies per iPSC splitting instructions.
2. Aspirate off old media with scratched colonies. Wash 1X with PBS to rinse lingering cells. Aspirate PBS.
3. Add 1mL iPSC media to well. Use scraper to "dissociate" colonies from well. ***Pipette media up and down in stripette to prevent colonies from sticking to the stripette walls. Pipette cell suspension to a 15mL conical tube. Rinse well with additional 1mL media.
4. Clusters should be roughly twice the size as clusters for passaging iPS cells.
5. Allow the iPS cell clusters to settle to the bottom of the tube (2-3min). Aspirate off the medium with caution so as to not aspirate the entire cell clump.
6. Resuspend cells in iPS media (for 1-2 wells use 10-12 ml iPS medium in a T-25) and transfer to flasks. 3-6 wells should be cultured in a T-75 with approx. 40-50 mLs.

   Cell aggregates will initially look unhealthy from shock of separation from feeders. To speed cell recovery, feed for the first time within ~12 hours and replace most of the medium to remove debris. Switching cells to a new flask is also useful to remove MEF that may have attached during the first 12 hours.
7. Continue feeding with iPS medium, no exogenous factors every day for four days.

   ***When feeding, use a 5 ml pipette to gently pull aggregates up and then blow them back into the medium 2-3 times. This will help clean dead cells off the aggregate surface. Let the clusters settle to the bottom in a standing flask and aspirate off the medium.

   See the alternative procedures section for a discussion of iPS aggregates vs. embryoid bodies

Differentiating to Primitive Neuroepithelia (Day 4-9)

After free-floating culture in iPS medium for 4 days, the aggregates are ready for further differentiation.

Reagents needed:

- Neural Induction (N2) media
- 15mL conical tubes
- T-25 or T-75 (non-cell culture treated – white caps) ***D5
- Matrigel-coated cell-culture 6wps ***D7

1. Let aggregates sink to the bottom of a flask. Aspirate old media and wash once with 5 mls of Neural Induction medium (no exogenous factors.) Aspirate wash media. Be careful not to suck up aggregates.
2. Resuspend cells in 10-12 (T-25 flask) or 40-50 (T-75 flask) mls of Neural Induction medium (N2) and transfer to a new flask.

   ***Cells in Neural Induction medium can be fed every other day.

3. After 2 days, aggregates should be bright and clear and are ready for attachment. Induce attachment by plating cells on a matrigel-coated plates. Matrigel-coated plated should be incubated for 1hr. ***See matrigel-coating protocol.
4. Aggregates should be transferred to a 15 ml tube and agitated gently with a 5 ml pipette to remove loose cells. Aspirate off old media. Resuspend aggregated in fresh Neural Induction (N2) media. 20-30 aggregates should be deposited in fresh Neural Induction medium in each well of a 6 well plate or 2-4 aggregates /coverslip.

   ***When plating aggregates, provide enough space for colonies to grow out without contacting one another.

5. If plating on a 6 well-plate, shake the plate on the incubator shelf up and down twice and then left and right twice gently to evenly distribute the clusters, the same method as for splitting iPS cells. Cells should attach overnight (minimize jarring plates, including frequent incubator closing and opening to improve attachment; an alternative method for attachment is to add 10% FBS in neural induction medium for overnight).
6. Attached aggregates will collapse to form a monolayer colony after 1-2 days. Continue feeding with Neural Induction medium (no exogenous factors) every other day.
7. After 10-11 total days of differentiation (4-5 days following attachment), over 95% of the colonies should take on a morphology in which the center cells exhibit an elongated, columnar morphology. *After 10-11 days of differentiation, primitive neuroepithelia is receptive to neural patterning signals. Attempts to add patterning signals (notably retinoic acid) prior to this time point can lead to differentiation to non-neural fates. At this point cells can continue to be cultured in Neural Induction medium or alternatively switched to conditions designed to regionally specify the neuroepithelia to more specific cell fates (such as the addition of SHH for ventralization).*

Generating Definitive Neuroepithelia (day 10-16)

1. ***If you see non-neuronal colonies that do not contain any primitive neuroepithelia cells (this should be less than 10% of colonies, see the troubleshooting section for additional help if this is not the case), scratch them off prior to media changes.
2. Neuroepithelial cells should be fed with the same medium every other day and cultured for 7 days (no exogenous factor). During this period starting at day 14-15 the columnar neuroepithelia cells will further compact and proliferate, often forming ridges or rings of cells outlining a distinct lumen. The overall morphology is reminiscent of the neural tube and cells at this stage are often referred to as neural tube-like rosettes.
3. After 17-18 days of differentiation under these conditions the neuroepithelia that makes up the rosettes will stain positive for the definitive neural tube stage marker Sox1 (dilute at 1:500).

Isolating Definitive Neuroepithelia (day 16-17)

To increase the purity of neuroepithelia cells generated, we have added a sub-culture step after the formation of neural tube-like rosettes (see notes on neuroepithelia isolation timing in the alternative procedures section).

- Neural Induction (N2/B27) media
- 15mL conical tubes
- T-25 or T-75 (non-cell culture treated – white caps)
- Rosette Selection Reagent
- cAMP/IGF-1

1. Treat the culture with Neural Rosette Selection Reagent per the manufacturer's instructions (1mL per 6 well). Incubate at 37°C for ~1hr. See instructions for more details.
2. Once the neuroepithelial cells (rosettes) are detached, collect the neuroepithelial cell clusters in a 15 ml centrifuge tube.

   Use gentle pipetting during rosette isolation as the cells separate easily and it is best to not break clusters of neuroepithelia up much initially.
3. Spin at 100g for 2 min and wash once with fresh Neural Induction medium.
4. Aspirate the medium and resuspend the clusters of definitive neuroepithelia in 10-12 (T-25 flask) or 40-50 (T-75 flask) mls of Neural Induction (N2/B27) media.
5. Over the next 24 hours the rosette aggregates will roll up to form round spheres while any flat non-rosette peripheral cells will usually attach to the culture vessel. After this period rosette aggregates should be switched to a new flask with Neural Induction medium N2/B27 +cAMP (final concentration at 1 uM) and IGF (10 ng/ml).
6. After several days in Neural Induction medium and B27, neuroepithelial aggregates (neurospheres) are ready for further differentiation to neural cells.

Differentiation of forebrain neurons from human iPS cells (day 24-28)

1. Collect the neurospheres in the flask to a 15 ml tube, centrifuge at 1000 rpm for 2 min
2. Wash once with DMEM-F12
3. Plate neurospheres on laminin/ Polyornithine (matrigel) coated coverslips (96-well plates) in the presence of Neural Differentiation medium supplemented with B27, cAMP (1 μM), and BDNF, GDNF, and IGF1(10ng/ml).
4. Please note: If the spheres are too big, they could be broken using glass pipette at 1 day before plating. Another way is to dissociate the neurospheres to small clusters using Accutase (2-5 minutes).
5. For specifying the ventral telencephalic progenitors, SHH (R&D, 100 ng/ml) will be added to the neuroepithelial cells at day 10 (day 10 to 24). After plating the neurospheres, cells are cultured in Neural Differentiation medium supplemented with cAMP (1 μM), and BDNF, GDNF, and IGF1 (10ng/ml). The amount of SHH is reduced to 10 ng/ml.

Alternative Procedures

Alternatives to laminin-enhanced adhesion: Attachment of iPS aggregates or neuroepithelial clusters can be accomplished with a variety of different adhesion molecules including fibronectin and Matrigel. Another quick and cost effective method to facilitate adhesion is to supplement

Neural Induction medium with 10% fetal bovine serum for 12-24 hours. The serum should then be washed away after the aggregates have attached. Although serum should be avoided for neuroepithelial differentiation, this short-exposure to enhance adhesion does not significantly reduce the overall efficiency of the culture system for generating neuroepithelia. It should be noted that the use of serum may affect some gene expression patterns.

Time for neuroepithelia isolation: Neuroepithelia cells can technically be isolated at any point after the primitive neuroepithelia stage at 10 days of differentiation and grown in suspension. The benefit of isolating neuroepithelia at the primitive stage is that it allows for early selection of neuroepithelia and limits cell death and differentiation that result from high density culture. However, culture of neuroepithelia as free floating clusters as opposed to a monolayer affects the exposure of cells to patterning signals and mitogens. If after 17-18 days of culture, neural tube-like rosettes are difficult to observe and there are too many non-neuroepithelial cells in culture, try enzymatically isolating neuroepithelia at the primitive neuroepithelial stage (day 10). Grow cells as aggregates for 1-2 days in Neural Induction medium and then re-plate the cells at a lower density. Take care to not break neuroepithelia cluster up too much. If neuroepithelia clusters attach and form monolayer colonies of larger flat cells like the ones seen at the edges of colonies at 10 days the clusters are too small. Breaking neuroepithelia clusters less initially or keeping clusters in culture longer will allow more cell proliferation and should solve this problem.

Mechanical neuroepithelia isolation: If you are having trouble enzymatically isolating neuroepithelia at the primitive (10 day) or definitive (17-18 day) stage from the flat surrounding cells, try isolating the neuroepithelia cells mechanically. Very gentle pipetting with a 1000 µl tip can usually dislodge the neuroepithelia which is denser in the center of colonies, as opposed to the flat, tightly bound cells at the periphery.

REAGENTS		
Item	**Supplier**	**Catalogue #**
L-Glutamine solution (200 mM)	Sigma, St. Louis, MO	G-7513
MEM non-essential amino acids solution	Gibco-BRL, Rockville, MD	11140-050
Kockout serum replacer	Gibco-BRL	10828-028
Dulbecco's modified eagle medium: Nutrient mixture F-12 1: 1 (DMEM/F12)	Gibco-BRL	11330-032
Dulbecco's modified eagle medium (DMEM)	Gibco-BRL	11965-092
Neurobasal medium	Gibco-BRL	21103-049
β-Mercaptoethanol (1000x)	Invitrogen	21985023
N2 supplement	Gibco-BRL	17502-048
Laminin from human placenta	Sigma	L6274
Bovine serum albumin (BSA)	Sigma	A-7906
Cyclic AMP	Sigma	D-0260
Sonic hedgehog (SHH)	R&D	1845-SH
Dispase	Gibco-BRL	17105-041
Acutase	Innovative Cell Technologies	AT104
Trypsin Inhibitor (1mg/ml dissolved in DMEM/F12 & sterile filtered)	Gibco-BRL	17075-029
Heparin	Sigma	H3149
Recombinant human BDNF	PeproTech Inc	450-02
Recombinant human GDNF	PeproTech Inc	450-10
Recombinant human	PeproTech Inc	100-11

IGF1		
Polyornithine	Sigma	P-3655
Fetal bovine serum	Sigma	F-2006

iPS cell Medium **500 ml**		
Component	**Amount**	**Final Concentration**
DMEM/F-12	392.5 ml	
Serum Replacer (KOSR)	100 ml	20%
MEM Non-Essential Amino Acids Solution	5 ml	0.1 mM
Beta-Mercaptoethanol (14.3 M)	3.5 µl	0.1 mM
L-Glutamine (200 mM)	2.5 ml	1 mM
Sterile filter with a 0.22 µM filter, add 4ng/ml bFGF just prior to feeding cells, Medium is stored at 4°C for up to 2 w.		

Neural Induction Medium (N2) **500 ml**		
Component	**Amount**	**Final Concentration**
DMEM/F12	490 ml	
N2	5 ml	1X
MEM Non-Essential Amino Acids Solution	5 ml	0.1 mM
Heparin (2 mg/ml)	500 µl	2 µg/ml
Sterile filter with a 0.22 µM filter, add cytokines and signaling molecules (such as FGFs) just prior to feeding cells.		

Neural Induction Medium (N2/B27) **500 ml**		
Component	**Amount**	**Final Concentration**
DMEM/F12	480 ml	
N2	5 ml	1X
B27	10 ml	1X
MEM Non-Essential Amino Acids Solution	5 ml	0.1 mM
Heparin (2 mg/ml)	500 µl	2 µg/ml
Sterile filter with a 0.22 µM filter, add cytokines and signaling molecules (such as FGFs) just prior to feeding cells.		

Neuronal Differentiation Medium **500 ml**		
Component	**Amount**	**Final Concentration**
Neurobasal medium	480 ml	
N2	5 ml	1X
B27	10 ml	1X
MEM Non-Essential Amino Acids Solution	5 ml	0.1 mM
Sterile filter with a 0.22 µM filter, add cytokines and signaling molecules just prior to feeding cells		

Dispase solution 10 ml		
Component	**Amount**	**Final Concentration**
Dispase	10 mg	1 mg/ml
DMEM/F12	10 ml	
Leave in a 37°C water bath for 15 min and filter sterilize the dispase solution with a 50 ml-Steri-flip before use.		

NE cells could be isolated by treated the culture with 0.5 mg/ml dispase in DMEM/F12 or Neural Induction medium. Incubate at 37°C ~2-3 minutes, monitoring closely for when inner neuroepithelia start to peel away from the flat peripheral cells at the edges of colonies.
Once the rosettes start to peel off, tap the plate to speed the process while trying to keep flat peripheral cells attached.

Mechanical neuroepithelia isolation: If you are having trouble enzymatically isolating neuroepithelia at the primitive (10 day) or definitive (17-18 day) stage from the flat surrounding cells, try isolating the neuroepithelia cells mechanically. Very gentle pipetting with a 1000 µl tip can usually dislodge the neuroepithelia which is denser in the center of colonies, as opposed to the flat, tightly bound cells at the periphery.