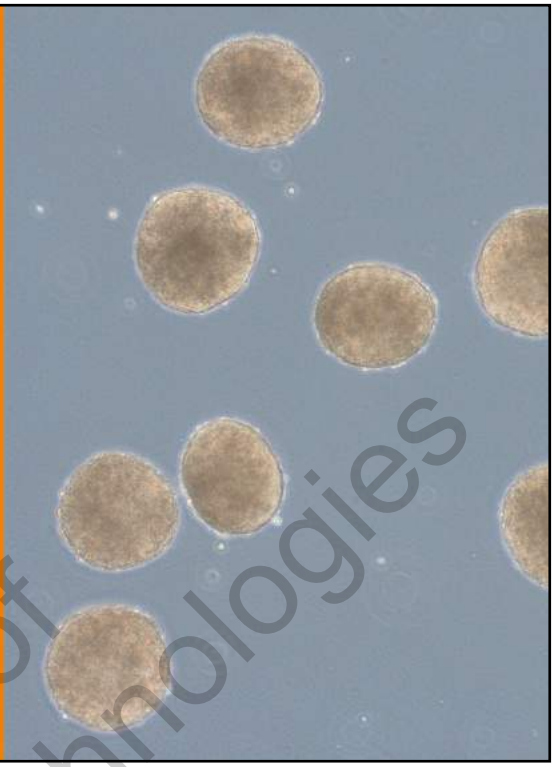
Neural Induction of hPSCs Using the Embryoid Body Method



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Scientist, Scientific Support, Neuroscience

STEMCELL™ TECHNOLOGIES

In this lecture, we will be going through the protocol for neural induction of human pluripotent stem cells using the embryoid body method.

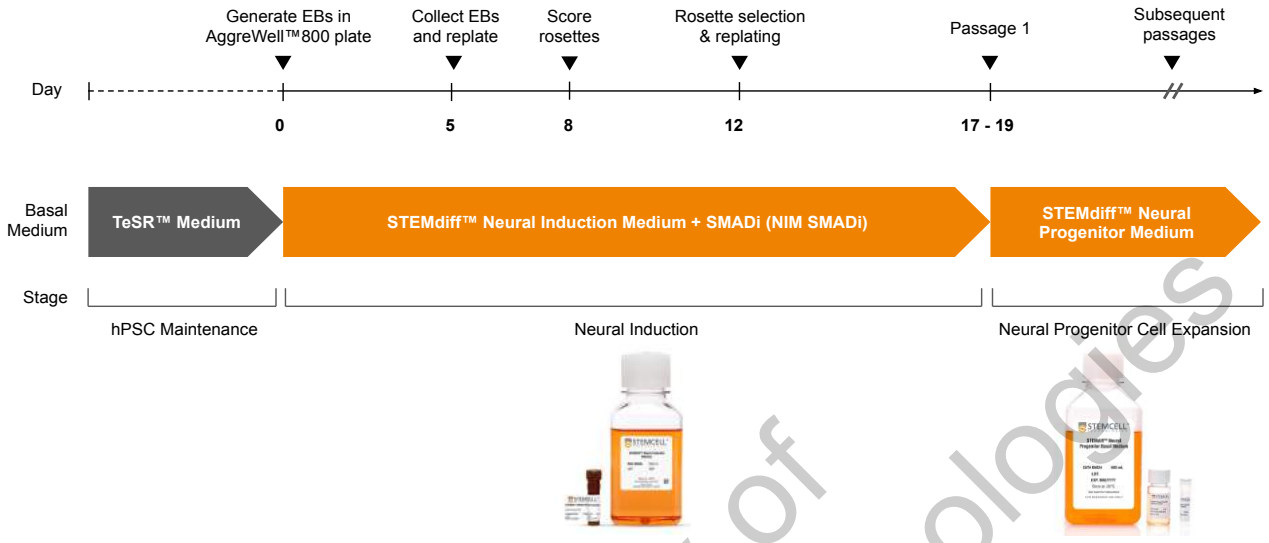
Learning Outcome

After this session, you will be able to:

- Confidently apply key techniques and methodological considerations for embryoid body (EB)-based neural induction of hPSCs to neural progenitor cells (NPCs).
- Describe morphological features of cultures undergoing EB-based neural induction.

After this session, you will be able to confidently apply key techniques and methodological considerations for embryoid body (EB)-based neural induction of hPSCs to neural progenitor cells (NPCs), as well as describe morphological features of cultures undergoing EB-based neural induction.

Neural Induction: Embryoid Body (EB) Protocol



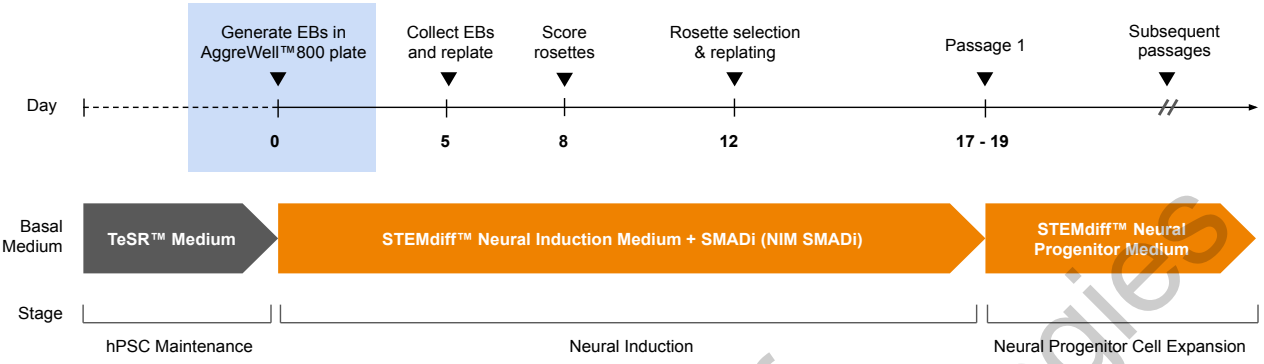
Here is a diagram of the embryoid body protocol for neural induction using the STEMdiff Neural Induction System. We will briefly review the main steps now, and then we will go through the individual key steps in more detail in the following slides.

The first step of the protocol is to generate embryoid bodies (or EBs) from human pluripotent stem cells (or hPSCs) that are being maintained in TeSR Medium. This step is done using AggreWell 800 plates, which we will describe in more detail shortly. At this point (Day 0), the cells are plated in the induction medium, which is STEMdiff Neural Induction Medium + the SMADi supplement. We call the complete medium NIM SMADi for short.

After 5 days, the EBs are collected and replated onto matrix-coated plates, which will allow them to attach and form neural rosette structures. These neural rosettes are scored on Day 8, and then selected and replated on Day 12. By Day 17-19 the neural induction is complete and the resulting neural progenitor cells (or NPCs) are ready for their first passage. At this passage, the cells are replated into STEMdiff Neural Progenitor Medium, which is specifically designed for maintenance and expansion of the NPCs.

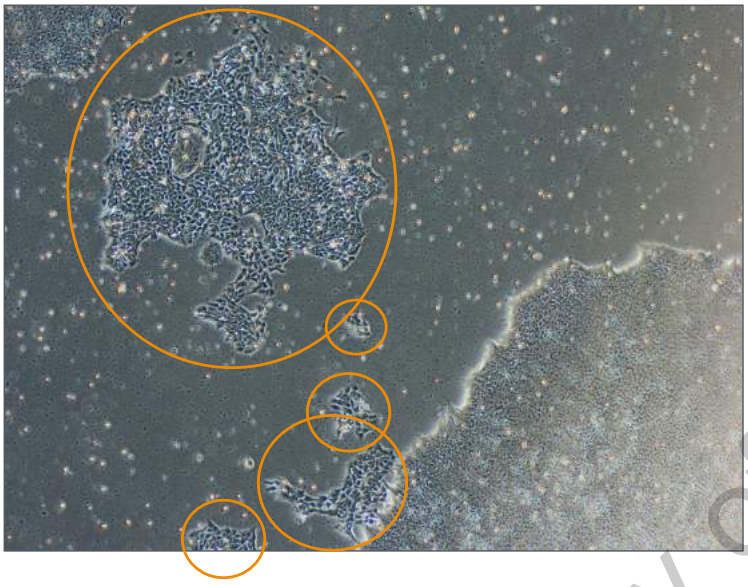
In the remainder of this lecture, we are going to walk through this protocol step-by-step to highlight key technical considerations and as well as expected culture morphology along the way. Following this lecture, you will also find a number of short laboratory videos within this session of the course. These videos will demonstrate how to perform each of these steps in the biosafety cabinet.

Day 0: Generate EBs



As mentioned, the first step of the protocol on Day 0 is to generate EBs from hPSCs using AggreWell 800 plates. This is done by first dissociating hPSCs growing as colonies in TeSR medium into a single cell suspension. However, before dissociating the hPSC cultures, it is very important to assess their morphology so that any areas of spontaneous differentiation can marked and removed prior to generating the single cell suspension.

Example: Marking Spontaneous Differentiation in hPSCs



Areas of spontaneous differentiation must be removed before dissociating hPSCs to single cells.

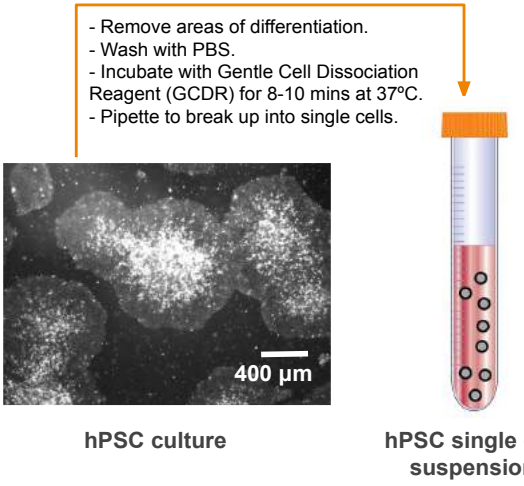
Key features of differentiation:

- Loose packing between cells
- Non-uniform colony borders
- Loss of high nuclear/cytoplasmic ratio

An example of this step is shown here. Key features of spontaneous differentiation in hPSC cultures include loose packing between cells, non-uniform colony borders, and (at higher magnification) loss of the high nuclear-to-cytoplasmic ratio characteristic of pluripotent stem cells. In the image, the areas circled in orange are displayed these key features of differentiation.

The culture dish should be scanned under the microscope for regions like this, and those areas can be marked on the underside of the plate with a marker so that they can be identified later on in the biosafety cabinet.

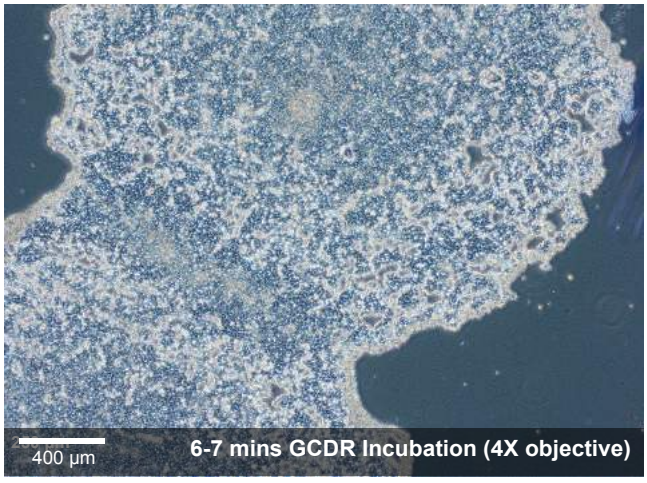
Uniform EB Formation Using AggreWell™800

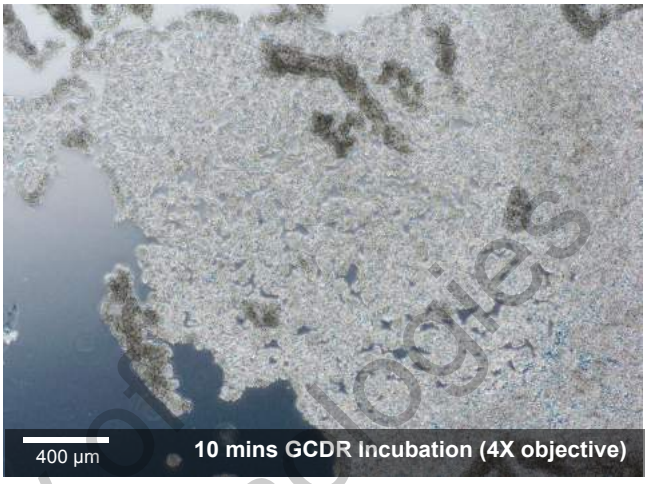


Once the starting hPSC cultures have been assessed and marked, those areas of differentiation are removed through scraping with a pipette tip or by vacuum aspiration. The culture medium is removed and replaced with Gentle Cell Dissociation Reagent (or GCDR), and then the plate is incubated for 8-10 minutes in the 37 degree CO2 incubator. This incubation time may need to be optimized slightly for each cell line.

Colony Morphology During 37°C GCDR Incubation

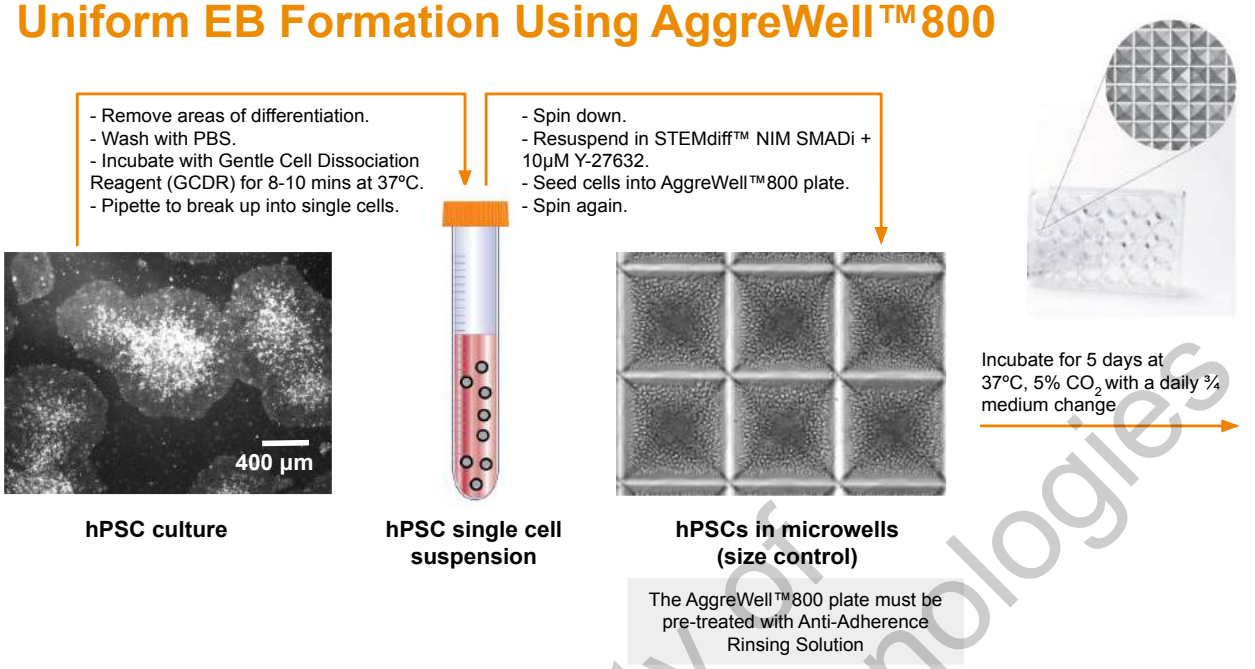
- Throughout the dissociation process, the edges of the colony will curl up and cells will separate and pull away from each other.





During this incubation period with GCDR, the colonies will gradually become broken up. On the left is an example of an hPSC culture about 6-7 minutes into the incubation period. The edges of the colonies are starting to curl up, and small gaps are beginning to appear near the edges of the colonies as the cells begin to separate and pull away from each other. At the end of the 10 minute incubation, on the right, gaps are visible throughout the entire colony. Clumps of cells may be seen breaking away from the main colony and floating in suspension.

Uniform EB Formation Using AggreWell™800



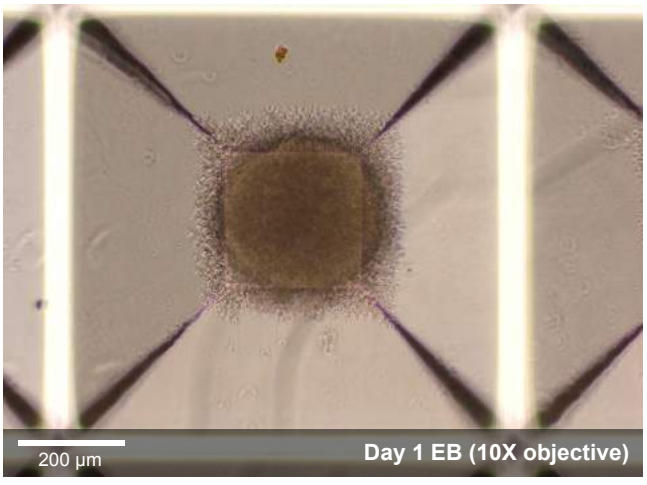
After the incubation, the cell suspension is pipetted up and down several times to dislodge any cells that are still attached to the plate, as well as to break up the cells into a single cell suspension. This suspension is centrifuged and then resuspended in STEMdiff NIM SMADi containing 10 micromolar of ROCK inhibitor Y-27632. ROCK inhibitor is included for this step because it promotes survival of hPSCs when they are in a single-cell format. The cell suspension is then seeded into a well (or wells) of an AggreWell 800 24-well plate, and the plate is centrifuged.

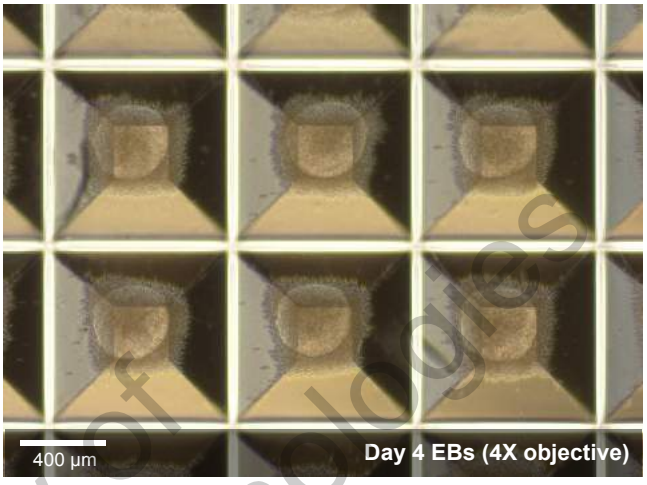
AggreWell 800 plates have inverted pyramidal-shaped microwells within each well. When hPSCs are seeded in the wells and the plates are centrifuged, it forces the cells to settle in the microwells with very uniform distribution, which will ultimately result in very high uniformity in the size of the resulting EBs.

Importantly, the AggreWell 800 plate must be pre-treated with Anti-Adherence Rinsing Solution before seeding the cells, to prevent them from adhering strongly to the plate itself. Over the next 5 days, the plate is incubated in the 37 degree CO2 incubator with a daily ¾ medium change with fresh STEMdiff NIM SMADi. By as early as 1 day into this 5-day incubation, the hPSCs in each microwell will self assemble together to form an embryoid body.

Representative EB Morphology

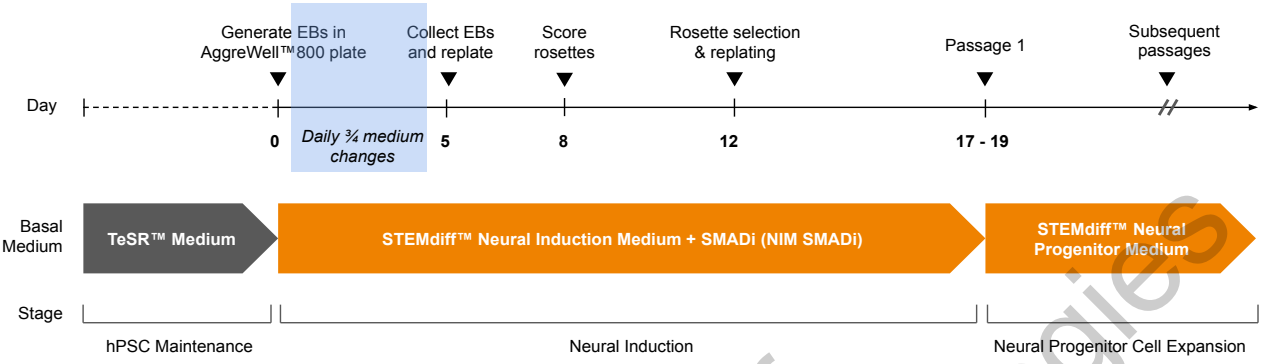
- Good EB morphology = round and symmetrical, partially translucent, smooth and defined edges, uniform
- Some cells may remain unincorporated





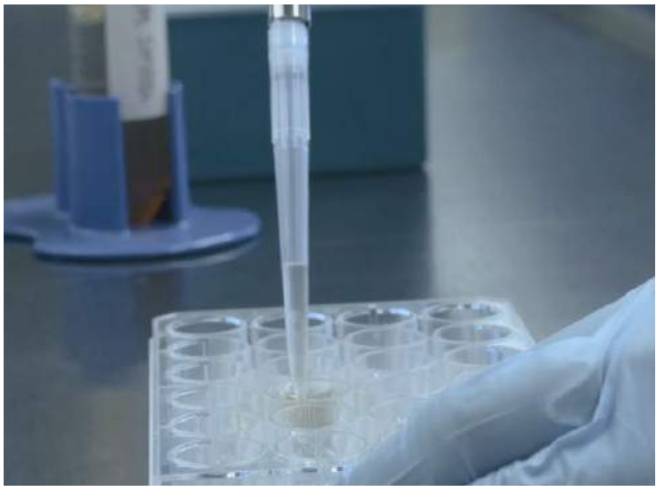
Representative morphology of EBs can be seen here at Day 1 and again at Day 4. An EB with good morphology is round and symmetrical, partially translucent, has smooth/defined edges, and is uniform in size compared to the other EBs. If the majority of EBs in the AggreWell plate do not have these characteristics, it is an indication that the hPSC quality was suboptimal, or that the protocol conditions are not ideal, and it is recommended to restart the experiment. As can be seen in these images, it is normal for some cells to remain unincorporated, and these may be visible as a layer underneath the EB.

Day 1 - 4: Daily ¾ Medium Change



During the 5-day EB stage, a daily ¾ medium change is required in order to replenish the medium components.

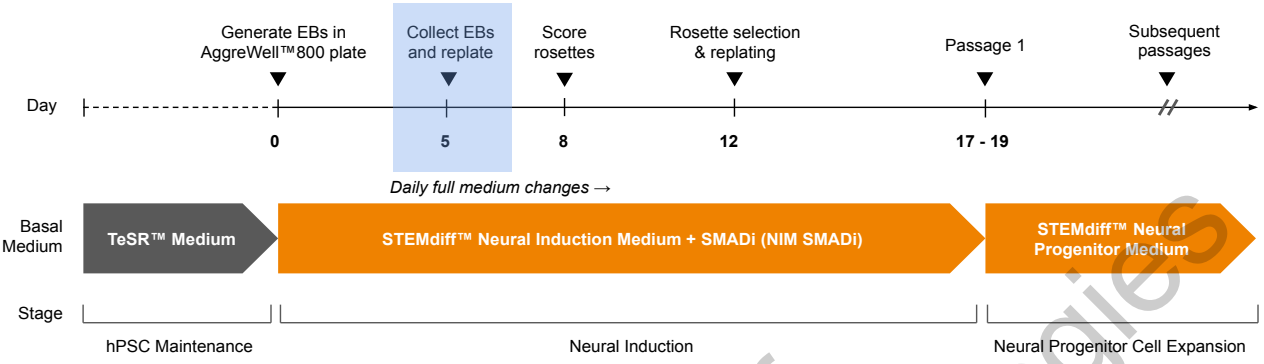
Daily ¾ Medium Change in AggreWell™800



- Remove and replace 1.5 mL of STEMdiff™ NIM SMADi medium **very carefully and slowly** using a 1000-µL pipettor.
- If EBs are disturbed they may jump out of the microwells and fuse together.
- Steady the pipette tip against the side of the well, keeping the tip just below the medium surface at all times.

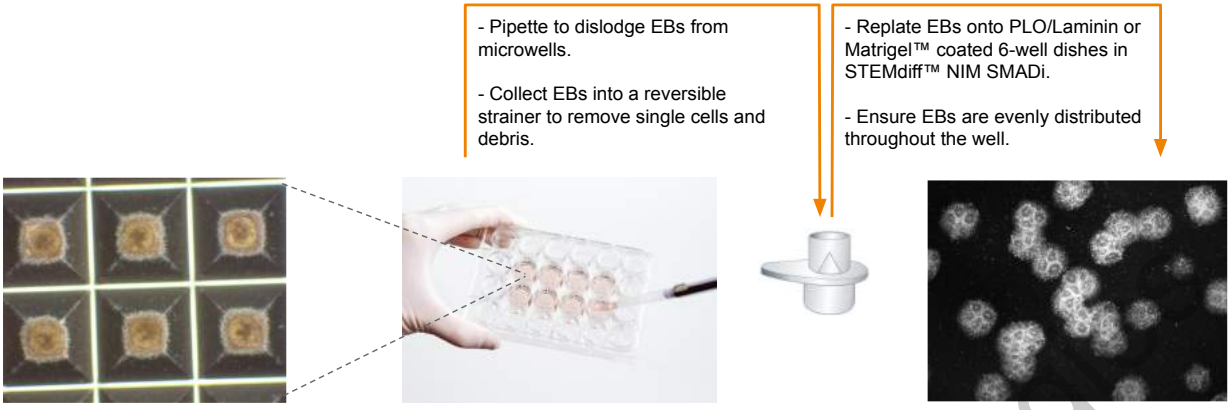
To perform this medium change, the first step is to carefully remove 1.5 milliliters of the existing medium using a 1000 uL pipettor. Then, 1.5 mL of fresh NIM SMADi medium is added back to the well. It is very important to perform this step gently and slowly, because if the EBs are disturbed, they may jump out of the microwells and fuse together. The best way to avoid this is to steady the pipette tip against the side of the well, and keep the very end of the tip just below the medium surface at all times.

Day 5: Collect EBs and Replate



After 5 days in the AggreWell 800 plates, the EBs are ready to be collected and replated into a matrix-coated plate, after which the cultures will continue to be maintained through daily full medium changes until the end of the induction period.

Harvesting EBs from AggreWell™800 and Replating

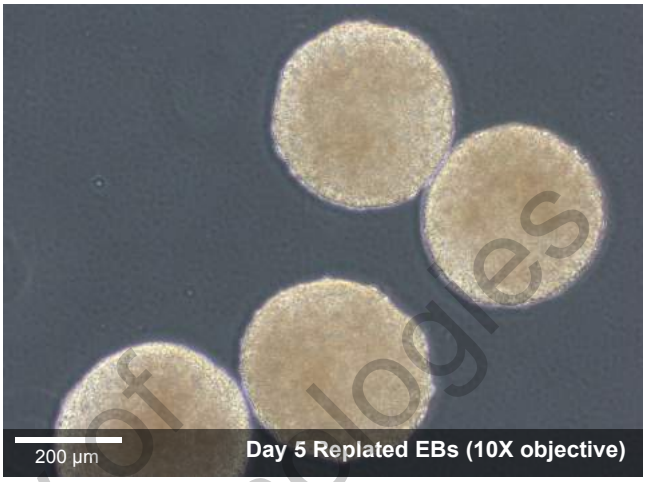


Continue with daily full medium changes with STEMdiff™ NIM SMADi for the rest of the induction

To collect the EBs, a wide-bore pipette tip must be used, so as not to damage them. The medium is pipetted forcefully to dislodge the EBs from the individual microwells. The suspension containing floating EBs is then passed through a reversible strainer to remove single cells and debris while keeping the EBs in the top compartment. The strainer is then inverted and the collected EBs are flushed using STEMdiff NIM SMADi into a 6-well plate that has been coated with either Matrigel or a combination of Poly-L-Ornithine and Laminin. At this point, the EBs should be distributed evenly throughout the well which will allow them to adhere down to the matrix with a uniform distribution over the following day. As early as 1 day later, neural rosette structures will become visible within the attached EB structures. Daily full medium changes with STEMdiff NIM SMADi are then performed for the rest of the induction protocol.

Representative Replated EB Morphology

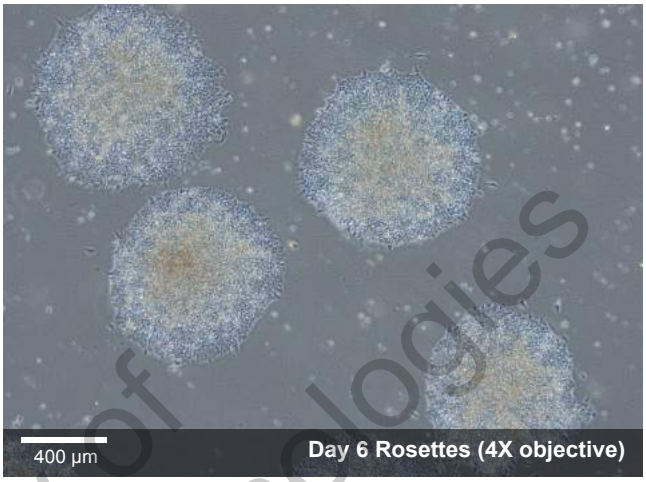
- EBs in suspension are still round and symmetrical, with distinct borders.
- Replated EBs appear uniform in size and shape.



These images depict representative EB morphology immediately after replating them from the AggreWell 800 plate on Day 5. The morphology should still be round and symmetrical, with distinct borders, and the EBs should appear uniform in both size and shape.

Representative Neural Rosette Morphology

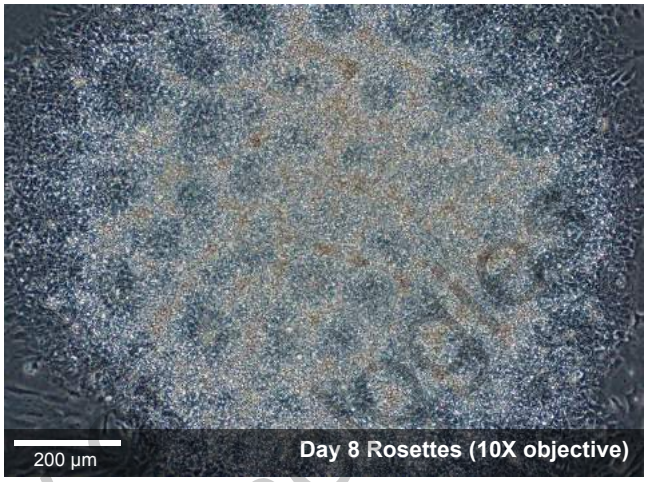
- After 1 day, replated EBs have attached and spread out, beginning to form rosette structures.
- Some debris is normal and can be removed with the daily medium change.



After 1 day, the replated EBs will have attached down to the culture substrate and spread out. They will begin to form rosette structures, as indicated by the circular patterns visible in these images. Some debris is normal at this stage, and can be removed with the daily medium change.

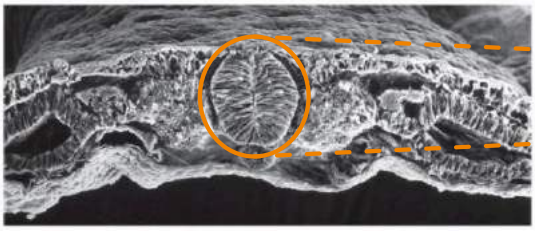
Representative Neural Rosette Morphology

- By Day 8, rosettes are clearly visible, consisting of tightly-packed, radially-arranged cells.
- Some ‘flat cells’ (neural crest cells, circled) can be seen starting to grow outwards between the rosettes.

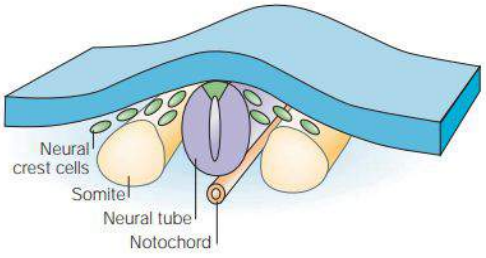


By Day 8, these rosette structures should be clearly visible, consisting of tightly-packed, radially-arranged cells. As indicated by the orange circle, some flat cells (which are likely to be neural crest cells) can be seen starting to grow outwards in the spaces between the rosettes. Neural crest cells are closely related to neural progenitor cells, but are considered to be an off-target cell type in this workflow.

Neural Rosettes: An In Vitro Equivalent of the Neural Tube

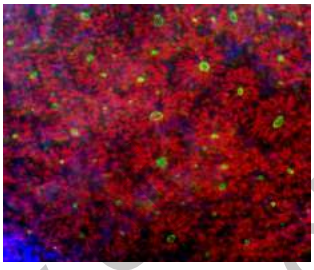


Gilbert SF, 2000. Developmental Biology 6e.

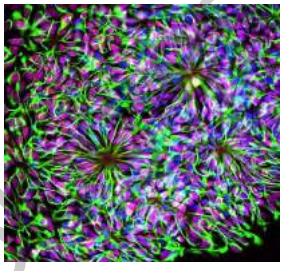


Gammill & Bronner-Fraser, 2003 *Nat Rev Neurosci.*

ZO-1/SOX1



Nestin/SOX1

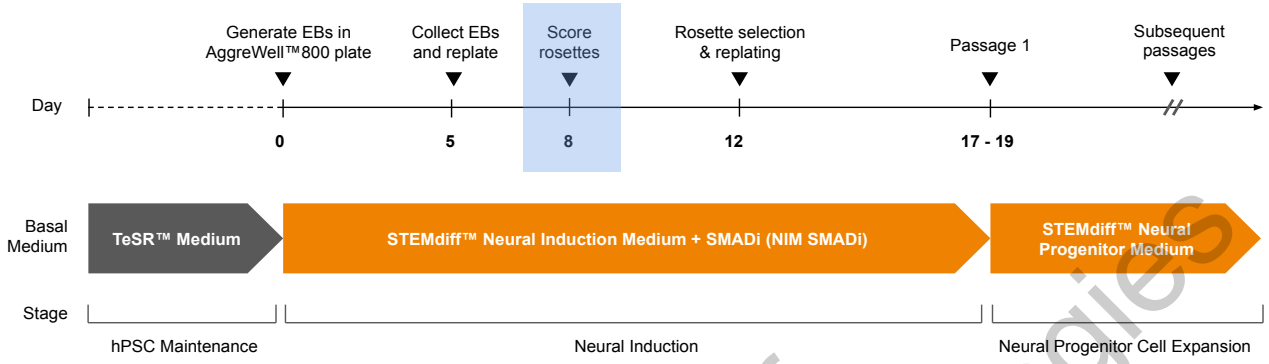


Not shown: Cells are also PAX6+

The emergence of these neural rosette structures and the flanking neural crest cells growing out from them is reminiscent of the in vivo process of neurodevelopment, and neural rosettes in culture can be considered an in vitro equivalent of the neural tube. If we were to stain for neuroectodermal markers such as Nestin and SOX1, we would see the rosette structures in these cultures staining positive for these markers, indicating the presence of central nervous system-type neural progenitor cells or NPCs. Although not shown here, these cells would also be expected to express the NPC marker PAX6.

Similar to how neural crest cells develop from the crest of the neural tube in vivo and migrate away during development, we also see this process being mimicked in the culture by the presence of neural crest cells proliferating away from those central rosettes.

Day 8: Score Neural Rosettes



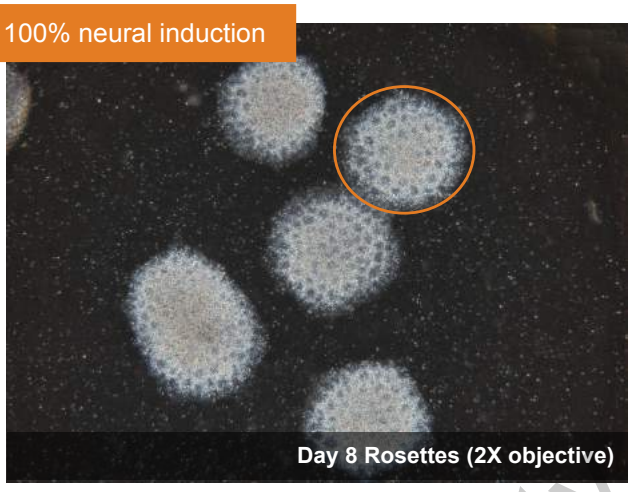
On Day 8, the cultures are ready for neural rosette scoring. This is an important visual checkpoint in the protocol that can give a good indication of how successful the differentiation has been so far.

Scoring Rosettes to Calculate Neural Induction Efficiency

$$\%\ Neural\ Induction = \frac{\#\ of\ EBs\ with\ \geq\ 50\%\ neural\ rosettes}{Total\ \#\ of\ EBs} \times 100$$

Neural induction score should be ≥ 75%

100% neural induction



Day 8 Rosettes (2X objective)

Why count at Day 8?

Counting too early:
Rosettes may not have fully formed yet

Counting too late:
"Flat cell" outgrowth may prevent accurate counting

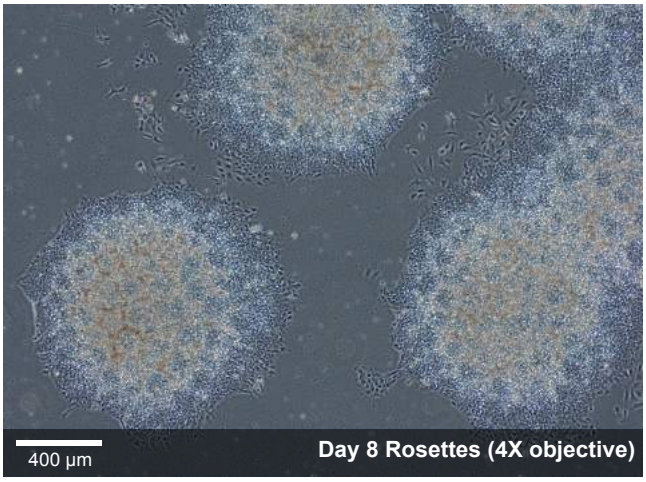
To calculate the Day 8 neural induction efficiency, first count the total number of attached EBs in a field of view under low objective magnification. An example of one single attached EB is circled here in orange. Next, count the number of those attached EBs that have at least 50% of their visible area filled with circular neural rosette structures. In the example shown here, all of the visible EBs would be counted, because they all contain rosette structures throughout nearly all of their visible area. Then, divide this number by the total number of EBs and multiply by 100 to obtain a percentage.

Ideally, this count will be performed over a few different fields of view to ensure that the calculated score is representative of the whole culture. As long as the neural induction score is at least 75% - that is - at least 75% of the attached EBs have neural rosette structures covering at least half of their area, the neural induction is considered to be successful thus far.

Why is this scoring step performed at Day 8? If the rosettes are scored too early, they may not have fully formed yet which can lead to underestimating the induction efficiency. In contrast, if the counting step is done too late, the outgrowth of flat cells may interfere with accurate scoring by making it more difficult to identify individual attached EBs..

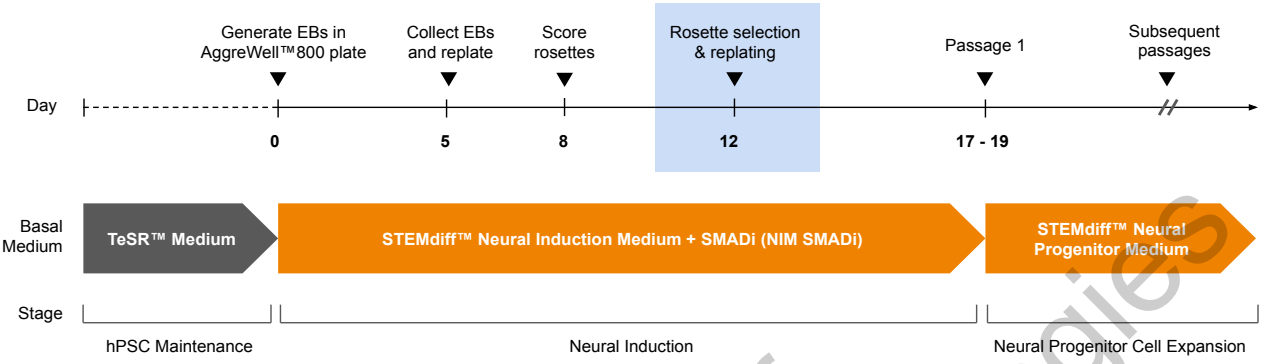
Representative Neural Rosette Morphology

- After Day 8, the ‘flat cells’ (neural crest cells) will continue to grow out from the rosettes.
- The rosettes should still be clearly visible and well-defined.



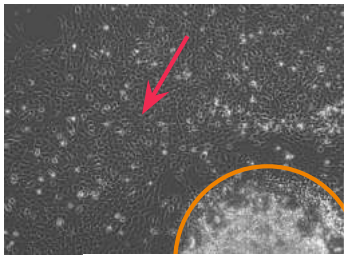
This can be seen in the representative images here, by comparing Day 8 to Day 10, where the outgrowth of flat neural crest cells appears can be seen to increase over time. The rosette structures should still be clearly visible and well-defined from the surrounding flat cells.

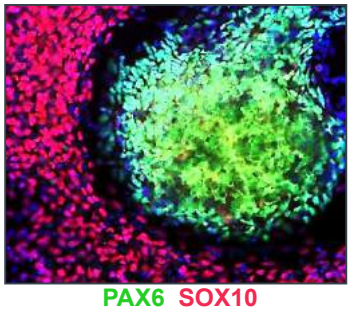
Day 12: Rosette Selection and Replating



On Day 12, the rosettes are selectively detached from the plate and replated onto a fresh matrix-coated plate for the remainder of the neural induction until Day 17-19.

Neural Rosette Selection with STEMdiff™ NRSR



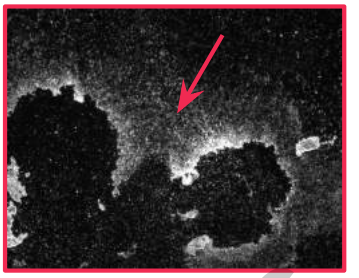


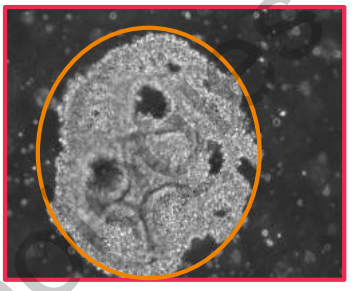
PAX6 SOX10

Incubate with STEMdiff™ Neural Rosette Selection Reagent (NRSR) at 37°C, 5% CO_2
NIM alone: 1 hour
NIM SMADi: 1.5 hours

Then, dislodge the rosettes by pipetting/rinsing them off. Spin down, and replate on fresh matrix-coated plates

It is better to **underselect** than to overselect.



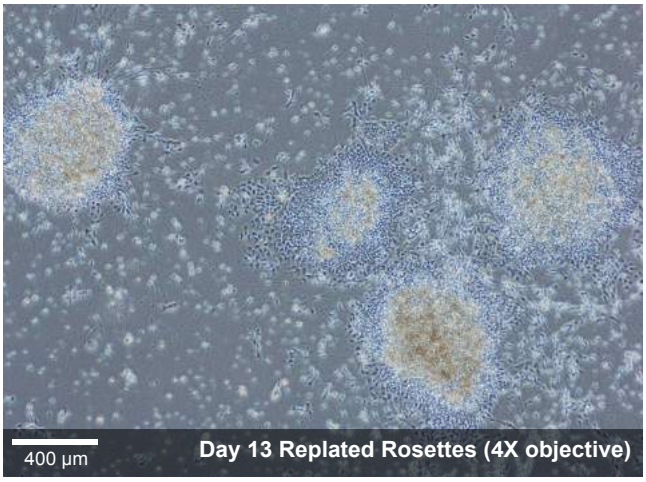


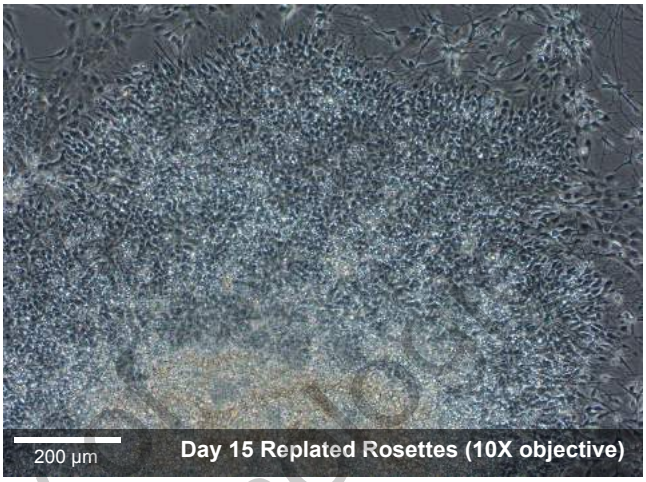
The purpose of this step is to selectively carry forward the rosette structures, which contain PAX6+ NPCs, while leaving behind the flat SOX10+ neural crest cells that grow out from them. This is done using the STEMdiff Neural Rosette Selection Reagent (or NRSR). The culture medium is removed and replaced with NRSR, and the culture is incubated at 37 degrees celsius, 5% CO2 for either 1 or 1.5 hours depending on whether SMADi is included in the induction medium. After the incubation, the rosettes are dislodged by pipetting and rinsing them off. Then the rosettes are centrifuged, resuspended, and plated onto fresh matrix coated plates. As before, the compatible matrices are either Matrigel or a combination of PLO and Laminin

The picture on the top right shows the original plate after the neural rosette selection. The contaminating neural crest cells can be seen to remain on the original plate while the rosette structures have been lifted off. An example of a lifted rosette clump can be seen below it. An important note for this step is that it is better to underselect than to overselect. In other words, is preferable to leave a few rosettes behind (sacrificing yield) than to unintentionally carry forward some of the off-target neural crest cells with the rosettes (sacrificing purity).

Representative Morphology After Rosette Selection

- By Day 13, rosettes have attached to the new plate, and Neural Progenitor Cells (NPCs) begin to grow out.
- Areas farther from the rosettes may appear sparse initially.

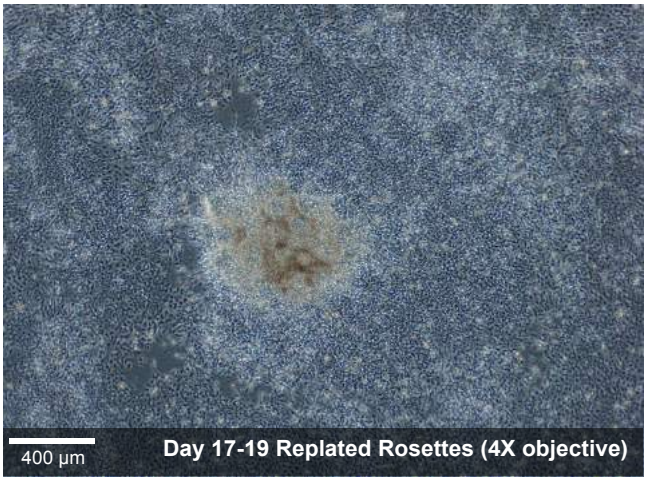


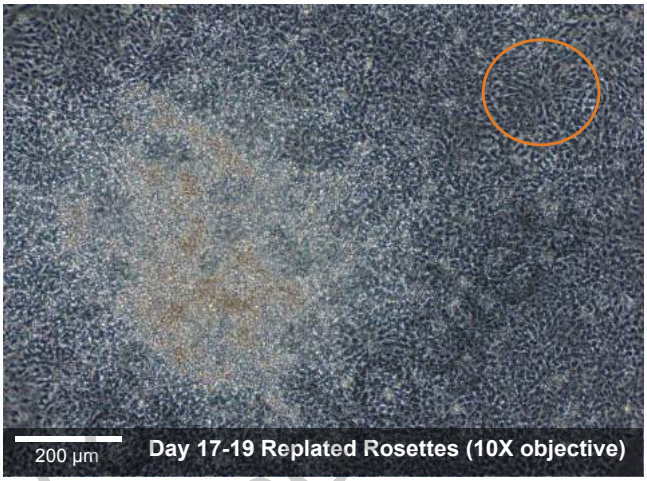


When the harvested rosette clumps are re-plated, they will attach down to the fresh matrix coating. As early as Day 13 (1 day after the rosette selection), the rosettes can be seen adhered to the plate, and neural progenitor cells (or NPCs) are beginning to grow out from them. Areas farther away from the rosettes may still appear sparse initially. A higher magnification view on Day 15 can be seen on the right, with densely-packed NPCs growing out as a monolayer from the edge of the re-attached rosette clump.

Representative Morphology After Rosette Selection

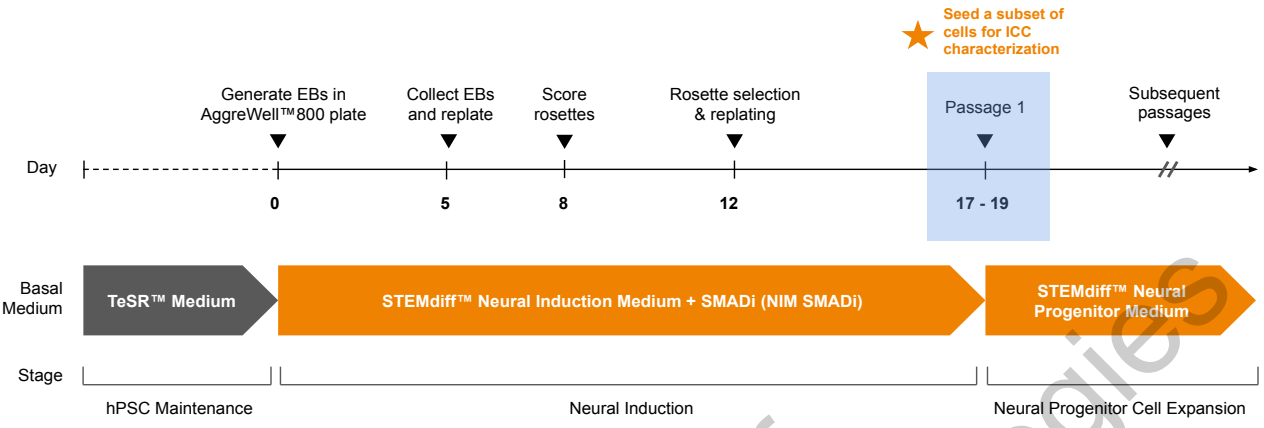
- By Day 17-19, NPCs have tightly filled most or all of the space between replated rosettes.
- Radial arrangements of NPCs may or may not be observed in the growing monolayer.





By Day 17-19, the NPCs will have continued to expand and tightly filled most or all of the space between the replated rosettes. Radial arrangements of NPCs may or may not be observed in the growing monolayer. An example of this is circled in orange. At this point, the cultures are ready for their first passage.

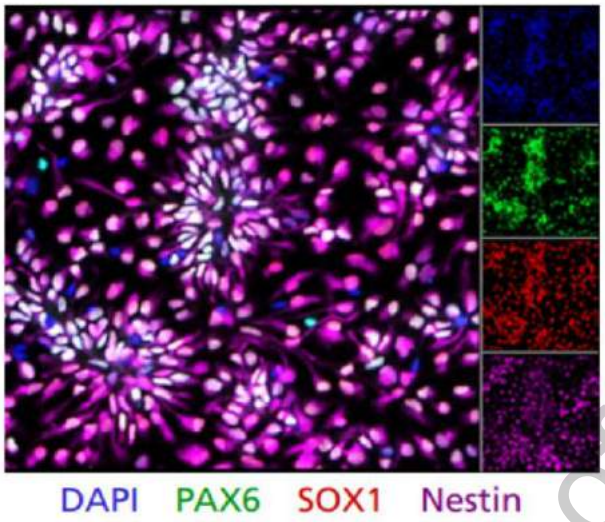
Day 17-19: Passage NPCs with ACCUTASE™



Expected Expansion = 3- to 5-fold per passage

For this first passage, the cells are dissociated into a single-cell suspension using ACCUTASE. The cells are then replated onto fresh matrix-coated plates in STEMdiff Neural Progenitor Medium, which is optimized for expansion and maintenance of NPCs for several passages. At this point, it is also recommended to seed a subset of the cells into a few wells of a separate plate (such as a 12- or 24-well plate) to perform immunocytochemical characterization of neural marker expression. This is a very important step to confirm that the neural induction was successful.

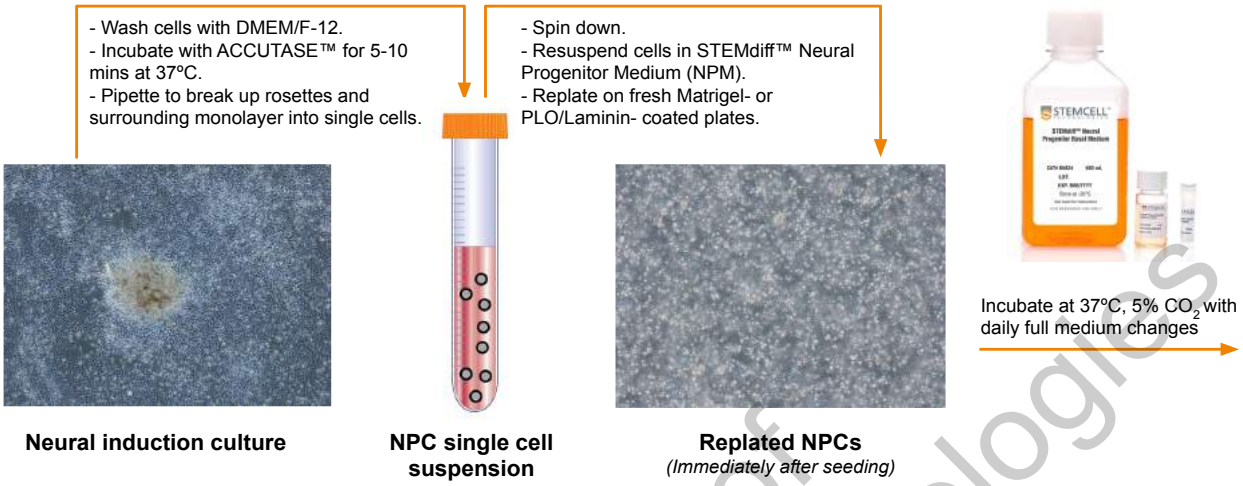
Neural Marker Expression: The Gold Standard



Successful neural induction:
>80-90% of cells expressing NPC markers at Day 17-19

The gold standard for confirming that the induction was efficient is to assess the expression of NPC markers such as PAX6, SOX1, and Nestin. In a successful neural induction experiment, these markers should be expressed in at least 80-90% of the resulting cell population by ICC analysis at the time of the Day 17-19 passaging step.

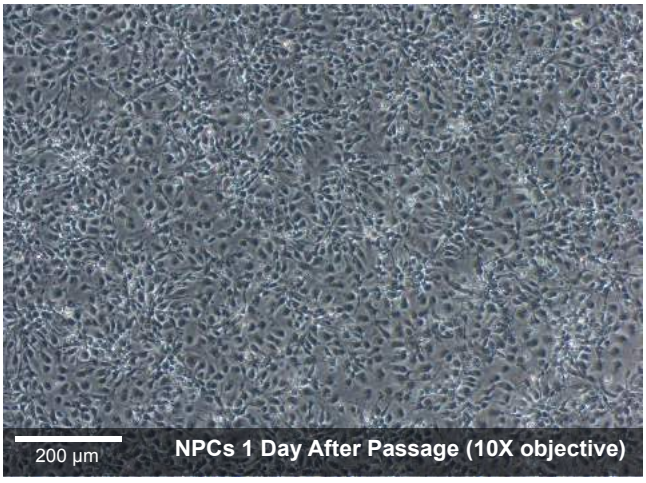
Passaging NPCs with ACCUTASE™

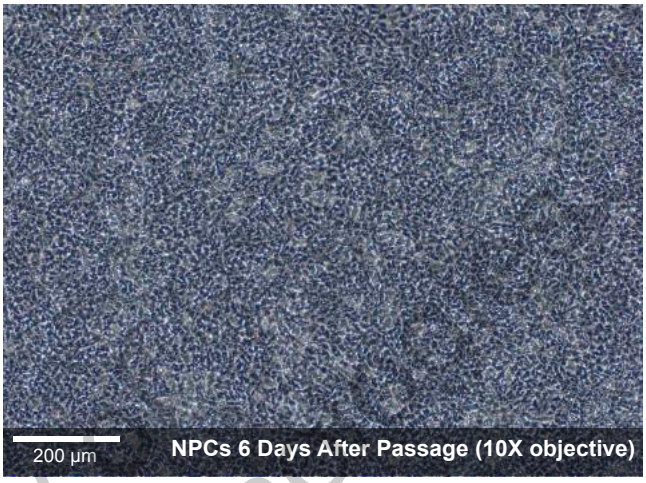


Specifically, the cells are first washed with DMEM/F12, and then they are incubated with ACCUTASE cell dissociation reagent for 5-10 minutes in the 37 degree incubator. After the incubation, the medium is pipetted up and down several times to break up both the rosette clumps and the surrounding monolayer into single cells. The cell suspension is centrifuged, resuspended in STEMdiff Neural Progenitor Medium (or NPM), and replated onto fresh Matrigel or PLO/Laminin coated plates. The cultures are they maintained in the incubator with daily full medium changes in STEMdiff NPM until they are ready for their next passage.

Representative NPC Morphology After Passaging

- NPCs appear dense soon after passaging, but can pack in more tightly with continued culture.
- NPCs have distinct, teardrop-shaped cell bodies, with minimal neuronal processes in the culture.

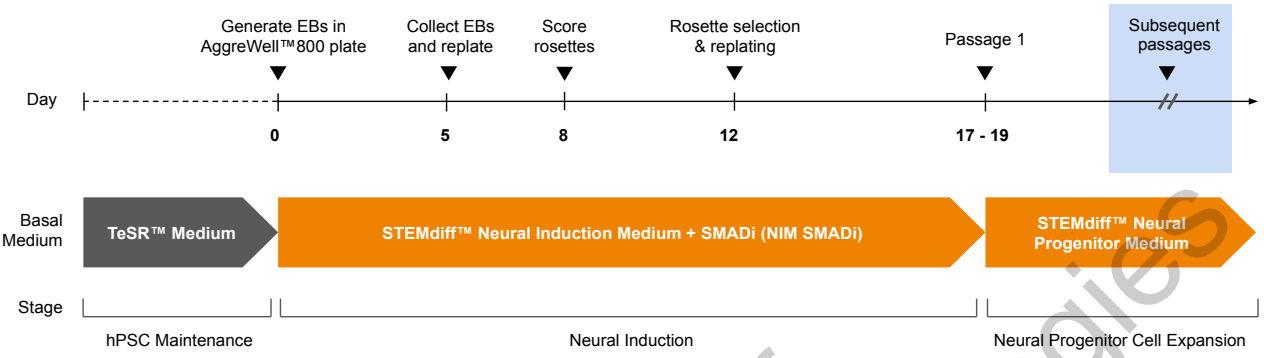




Representative NPC morphology after the first passage can be observed here. NPCs may appear quite dense soon after passaging, as shown on the left. However, the cells can pack in much more tightly together with continued culture, as can be seen in the picture on the right, taken 6 days after the passage. In terms of the individual cell morphology, NPCs have distinct, teardrop-shaped cell bodies. Only minimal neuronal processes should be visible in the culture.

The cultures are typically ready for passaging again about 5-7 days after the previous passage. The cells can become quite dense during this time, but they should still be healthy, which means the timing of these subsequent passages is a bit flexible.

Expansion & Maintenance of NPCs



Expected Expansion = 3- to 5-fold per passage

After this first passage, the NPCs may continue to be maintained in the STEMdiff Neural Progenitor medium which supports approximately 3- to 5-fold expansion of the total cell number per passage. Key things to keep in mind are that NPCs should be maintained at high densities to preserve their proliferative progenitor state, neural marker expression should be re-checked periodically after any extended passaging, and it is recommended to keep the number of passages in STEMdiff NPM to fewer than 5 if proceeding to neuronal differentiation downstream, as this will ensure best results

Summary

- The EB method for neural induction uses AggreWell™800 plates to generate highly uniform EBs, which are cultured in STEMdiff™ SMADi Neural Induction Medium to promote a neural fate.
- The rosette structures that form in the EB-based neural induction are in vitro equivalents of the developing neural tube in vivo.
- **Key steps of the EB neural induction protocol include:**
  - Starting with high-quality hPSCs that have had areas of spontaneous differentiation removed.
  - Checking for proper EB and NPC morphology throughout the differentiation.
  - Performing medium changes very slowly and carefully during the AggreWell™ stage.
  - Checking that the neural induction score on Day 8 is ≥75%.
  - Avoiding overselection of rosettes on Day 12.
  - Checking for positive expression of neural markers (>80-90% PAX6, SOX1, Nestin) at the end of the induction.

In summary, the EB method for neural induction uses AggreWell™800 plates to generate highly uniform EBs which are cultured in STEMdiff™ SMADi Neural Induction Medium to promote a neural fate. The rosette structures that form in the EB-based neural induction are in vitro equivalents of the developing neural tube in vivo.

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