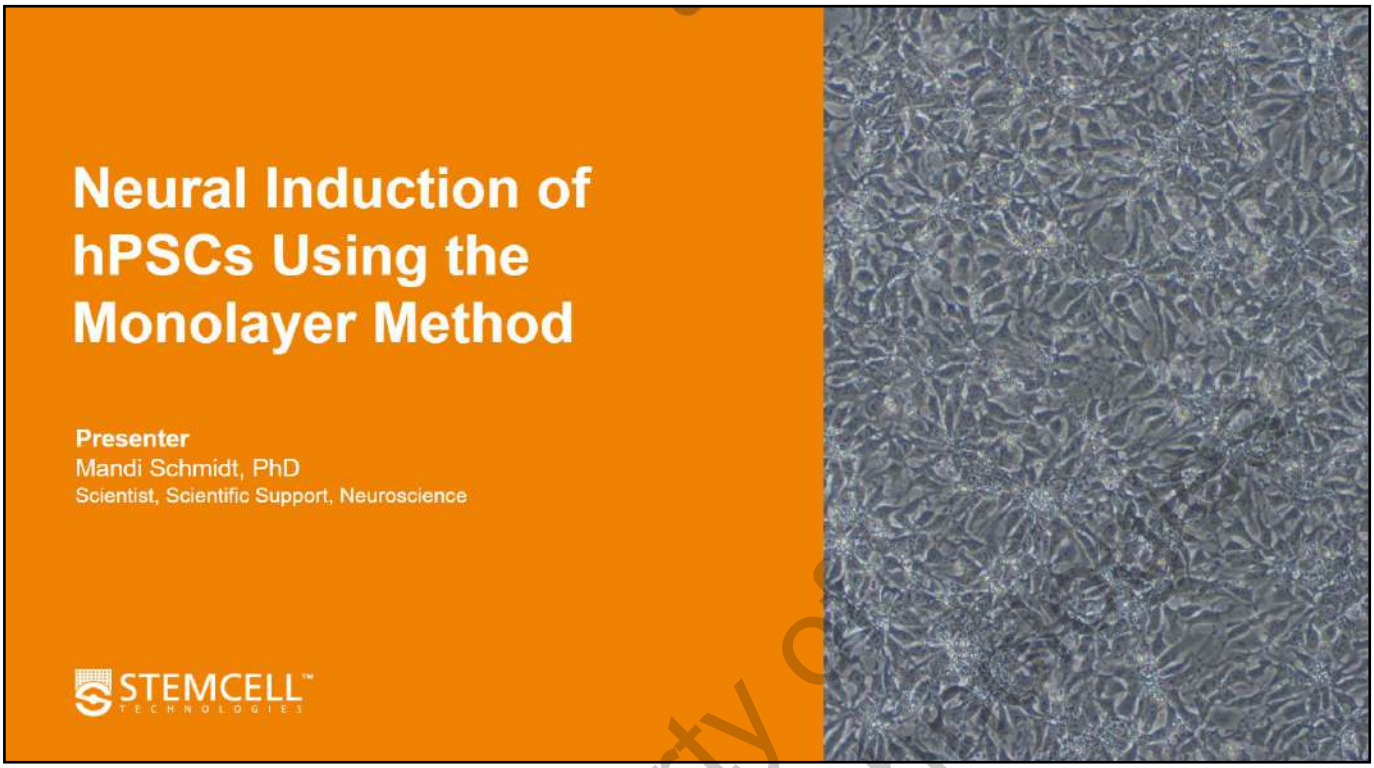


In this lecture, we will be going through the protocol for neural induction of human pluripotent stem cells using the monolayer method.

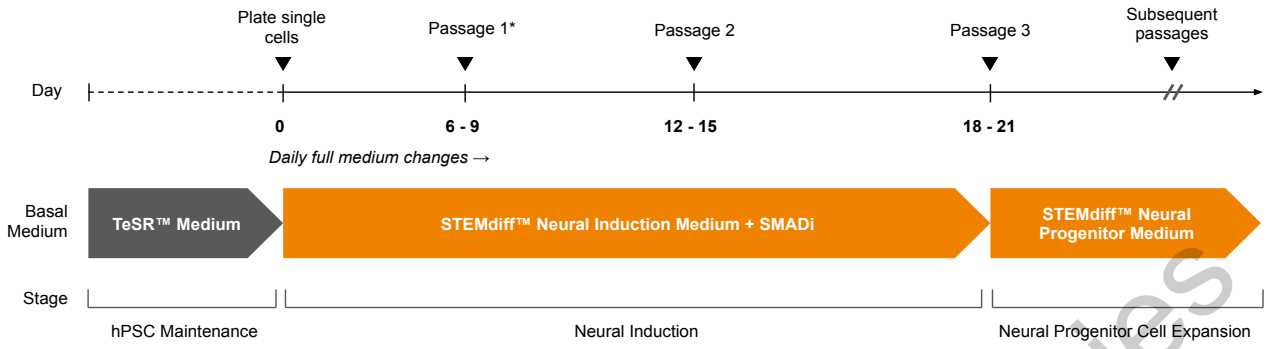
Learning Outcome

After this session, you will be able to:

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

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

Neural Induction: Monolayer Protocol



*By Day 6-9, NPC markers (nestin, PAX6, SOX1) should be highly expressed. However, a total of 3 passages in the induction medium are required to ensure NPCs are fully induced and well-established.

Here is a diagram of the monolayer 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.

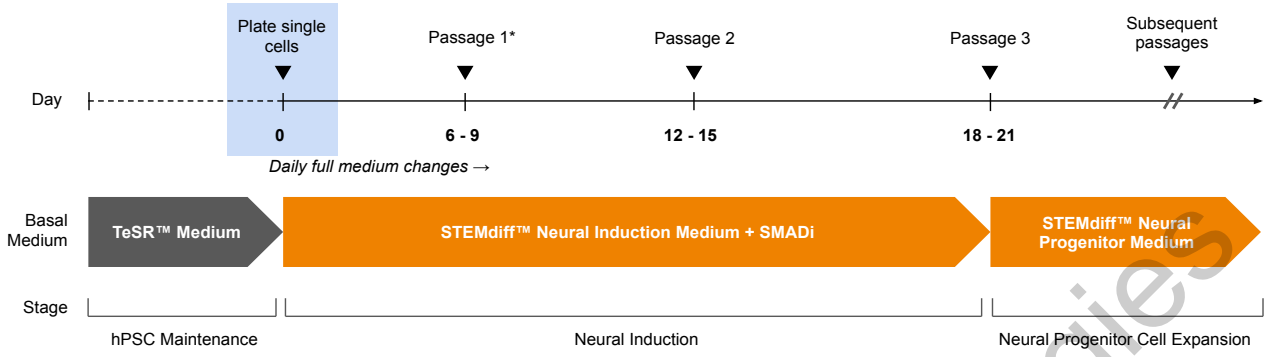
To initiate the culture, human pluripotent stem cells (or hPSCs) cultured in TeSR medium are dissociated and plated as single cells onto matrix-coated plates. At this point (Day 0), the cells are plated in the induction medium, which is STEMdiff Neural Induction Medium + the SMADi supplement. We call the complete medium NIM SMADi for short.

The cells are then maintained for two passages with daily full medium changes. On day 18 to 21, the resulting neural progenitor cells (or NPCs) are ready for their third passage, at which time they are replated into STEMdiff Neural Progenitor Medium, which is specifically designed for maintenance and expansion of the NPCs.

Later in this lecture, we will discuss characterization of the NPCs by assessing neural marker expression. Important to note for the monolayer neural induction protocol is that as early as Day 6-9 (i.e. at passage 1) these markers (such as nestin, PAX6, and SOX1) should already be highly expressed. However, a total of 3 passages in the neural induction medium are required to ensure that the NPCs are fully induced and well-established before moving on to expansion or downstream differentiation

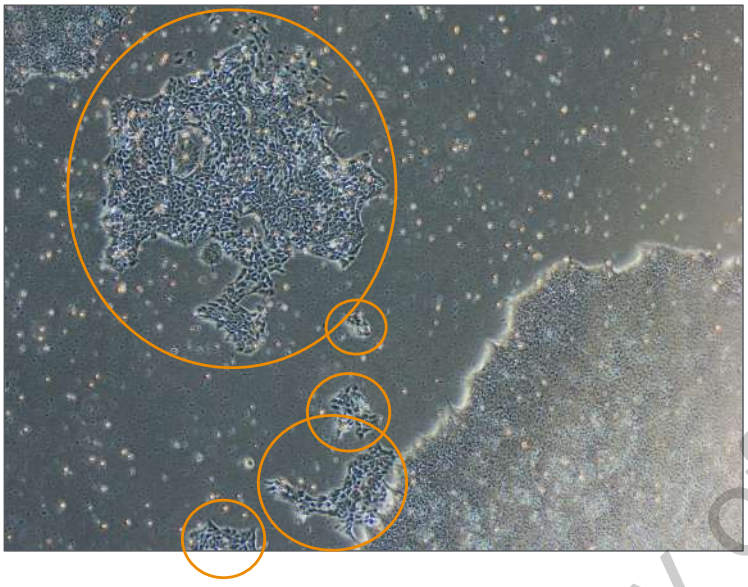
In the remainder of this lecture, we are going to walk through this protocol step-by-step to highlight key technical considerations and as well as expected culture morphology along the way. Following this lecture, you will also find a number of short laboratory videos within this session of the course.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

Neural Induction: Monolayer Protocol



As noted, the first step of the protocol on Day 0 is to generate a single cell suspension of hPSCs. Before dissociating the hPSC cultures, it is very important to assess their morphology so that any areas of spontaneous differentiation can be 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 with GCDR.

Key features of differentiation:

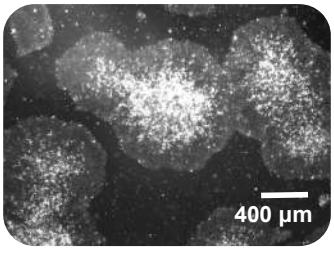
- Loose packing between cells
- Non-uniform colony borders
- Loss of high nuclear/cytoplasmic ratio

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.

Seeding hPSCs for Monolayer Neural Induction

- Remove areas of differentiation.
- Wash with PBS.
- Incubate with Gentle Cell Dissociation Reagent (GCDR) for 8-10 mins at 37ºC.
- Pipette to break up into single cells.



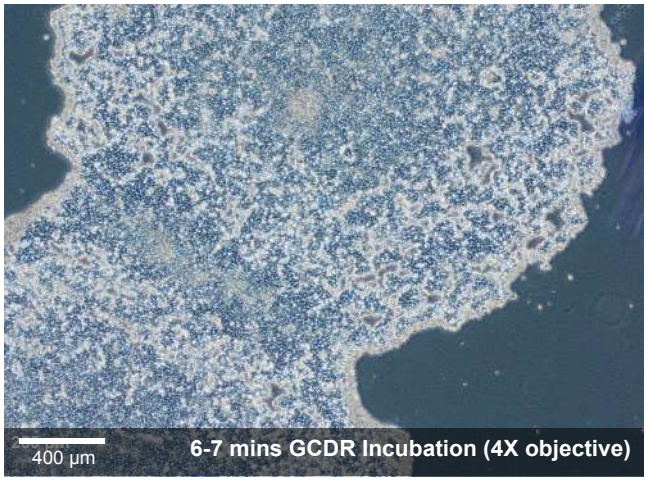
hPSC culture

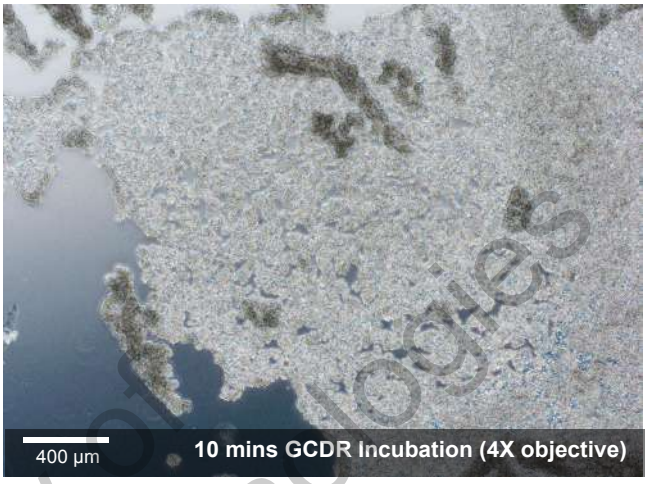
hPSC single cell suspension

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

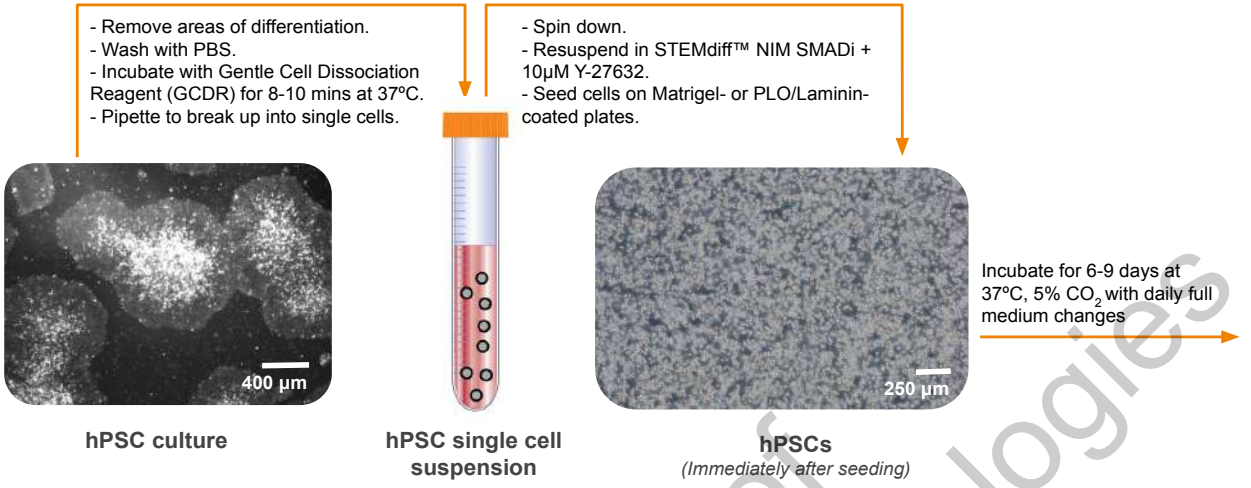
- Throughout the dissociation process the edges of the colony will curl up and cells will separate and pull away from each other.





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.

Seeding hPSCs for Monolayer Neural Induction



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 culture plates coated with Matrigel or a combination of Poly-L-Ornithine and Laminin.

The cells will appear very dense immediately after seeding. The plate is then incubated at 37 degrees and 5% CO2. Within the first few hours of seeding, the cells will begin to settle down and adhere to the plate surface as a monolayer. The cultures are then maintained for 6-9 days with daily full medium changes with fresh STEMdiff NIM SMADi until the first passage.

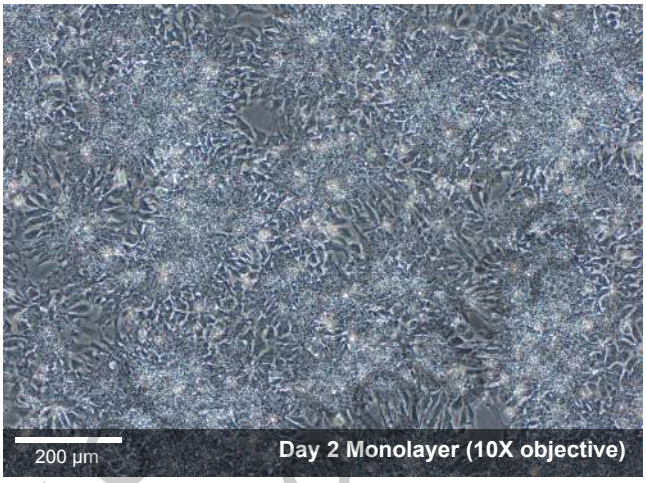
Representative Morphology - Monolayer Neural Induction

- Day 1: Cells may appear almost 100% confluent.
- Do not passage early.

Shown here are representative images from 1 day after seeding, taken at 4X and 10X objective magnification. As you may notice, these cells appear nearly 100% confluent already due to the high initial seeding density. Not only is this normal to observe, but this high density is ideal for good induction, because the molecular pathways involved in neural specification are highly reliant on cell-to-cell signaling. Furthermore, as you will see in the following images, NPCs can pack together even more tightly than what you see here, as they continue to divide. For this reason, the cultures should not be passaged early, but left until at least Day 6.

Representative Morphology - Monolayer Neural Induction

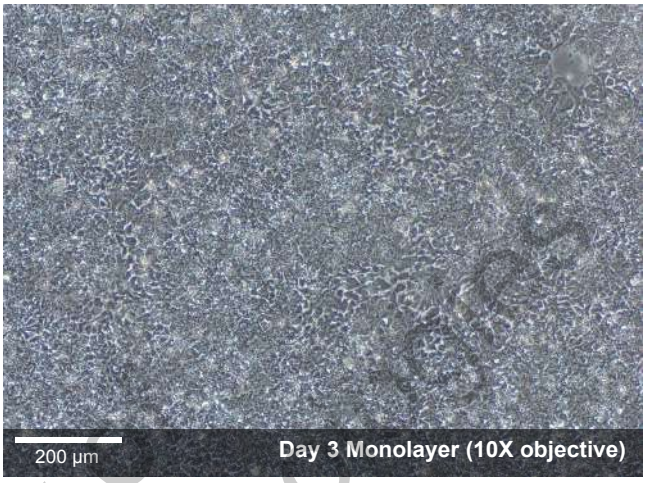
- Day 2: Heterogeneous culture morphology and some floating dead cells may be observed.



In this slide we're looking at the same cultures after another day of culture, on Day 2. You might notice at this point that the monolayer appears to be a bit less homogeneous - this is normal. It's also normal to see some floating dead cells and debris in the middle of the wells. However, the daily medium change with fresh STEMdiff NIM SMADi helps to remove these.

Representative Morphology - Monolayer Neural Induction

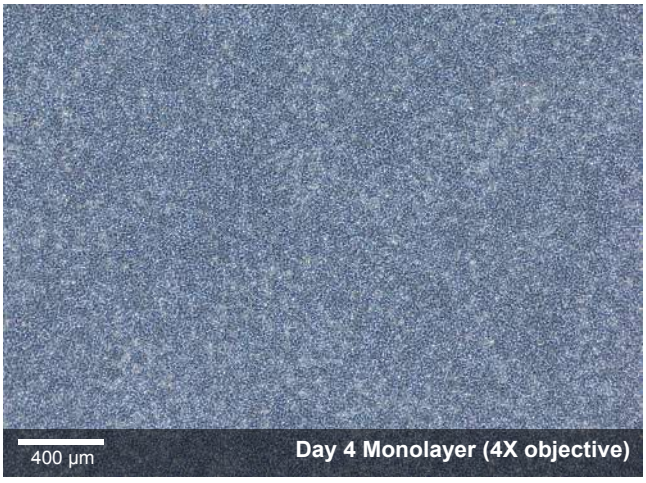
- Day 3: Culture grows more tightly packed as cells proliferate.

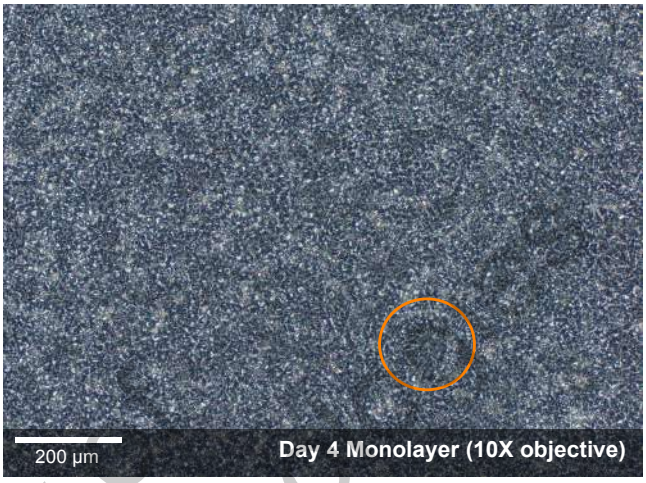


Here, we've jumped ahead to Day 3 of the protocol. In this culture, there is still a bit of an uneven distribution of the cells, where some areas are more sparse and others are packed in more tightly, but the culture is becoming even more dense as the cells proliferate.

Representative Morphology - Monolayer Neural Induction

- Day 4: Radial rosette-like patterns may be observed in the monolayer. Presence of this can vary between cell lines.

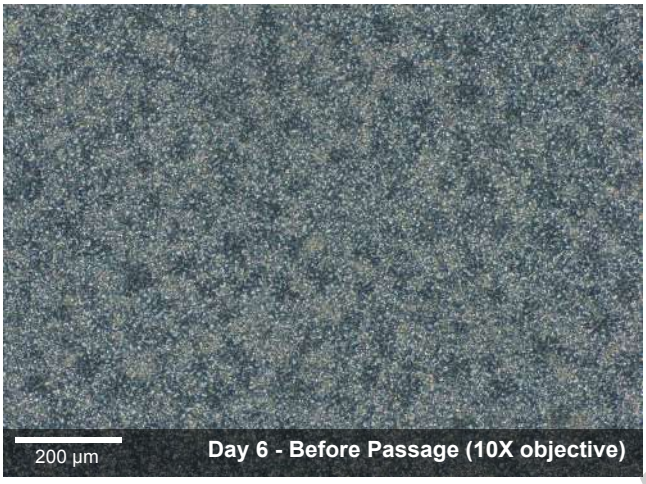


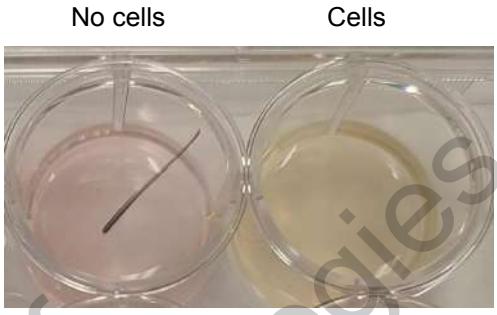


Moving one more day forward, we can see representative images from Day 4 here. Again, the cells are growing even more tightly packed. Something that may be observed in the culture are faint, radial, rosette-like patterns. One of them is outlined here in orange, where you can see that the cells have arranged themselves into a small circle in a polarized orientation. This feature will not always be obvious, as it can vary quite a bit between cell lines and experiments.

Representative Morphology - Monolayer Neural Induction

- Day 6: Culture density may make it difficult to identify individual cells.
- Medium may appear yellow because of the high metabolic rate of the culture.

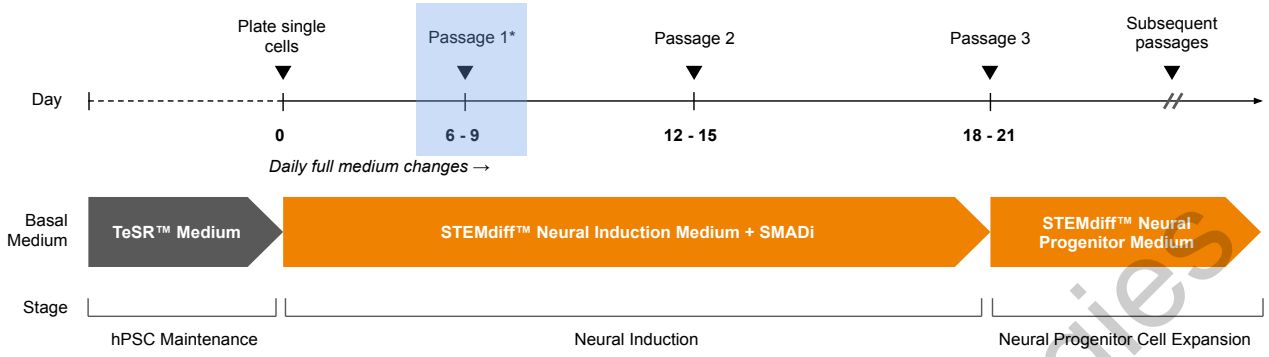




Finally, here you can see a representative image from Day 6 of that same culture. In this case, the culture was passaged on Day 6 and this image was taken immediately beforehand. As you may notice, the culture is incredibly dense at this point, which may make it difficult to identify individual cells. There still may or may not be the presence of radialized patterning throughout.

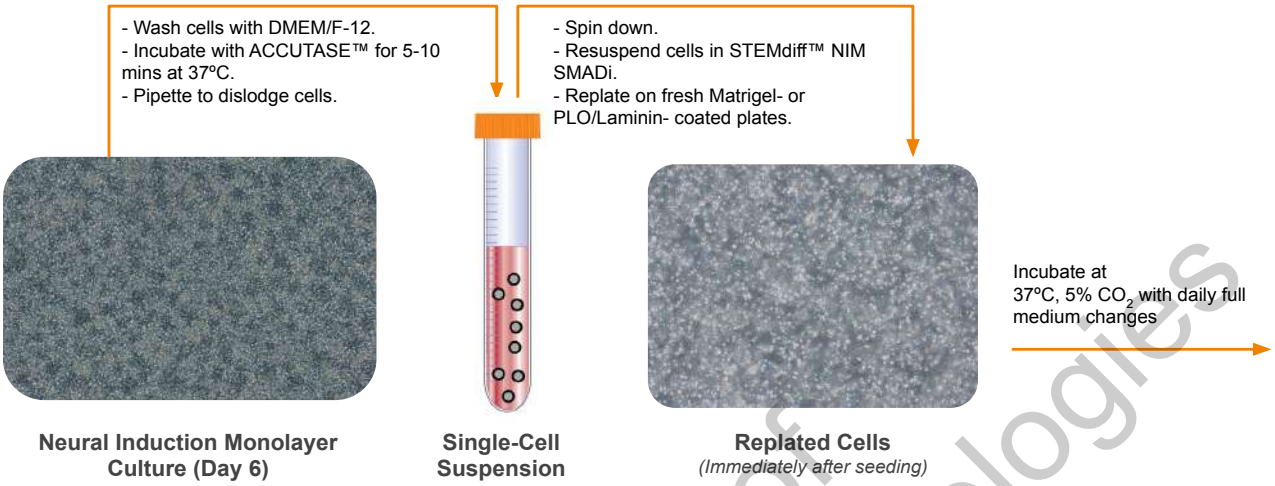
Shown on the right of this slide is an example of the medium color that may be expected prior to the daily medium change throughout these first 6 days. On the left we have a well that was incubated without cells. On the right, you may see that the medium color is quite yellow, and this is due to the high density and high metabolic rate of the culture. For this reason, it is very important not to skip any of the daily feeds, as these cultures can expend the medium quite quickly, meaning it needs to be replaced regularly to keep the cells healthy.

Neural Induction: Monolayer Protocol



By Day 6-9 the cultures will be ready for their first passage. This passage is done using ACCUTASE to lift the cells and dissociate them into a single cell suspension which is then re-seeded onto a fresh matrix-coated plate.

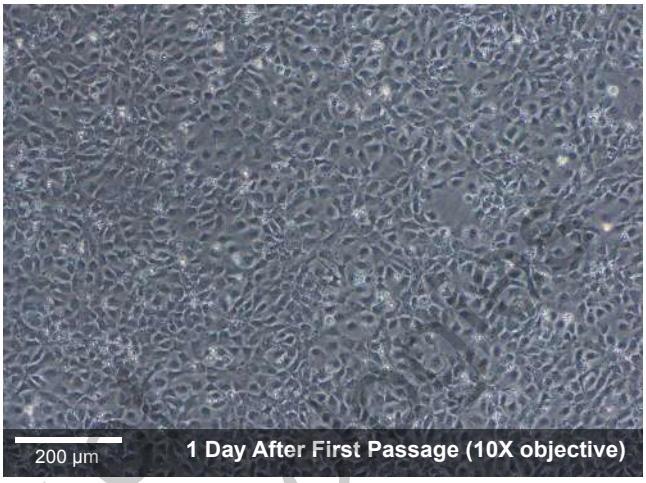
Passaging NPCs with ACCUTASE™



More specifically, to perform this passage, 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 the monolayer into single cells. The cell suspension is centrifuged, resuspended in fresh STEMdiff NIM SMADi, 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 NIM SMADi until they are ready for their next passage.

Representative Morphology - Monolayer Neural Induction

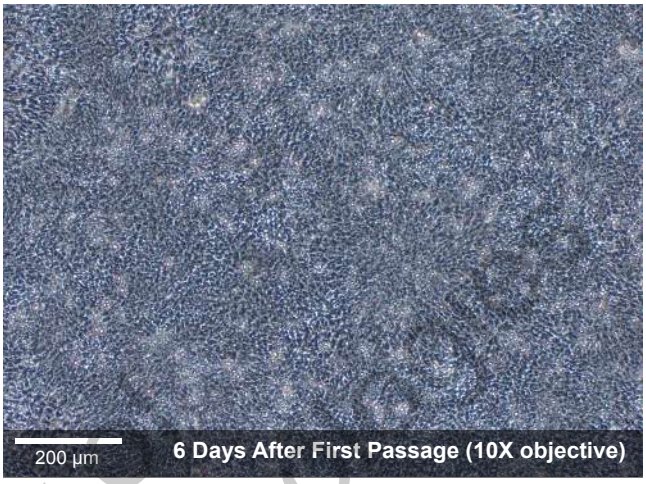
- 1 Day After Passage: Cells possess triangular or teardrop-shaped cell bodies that are distinct from one another and yet still closely packed.



Here you may see the morphology of the cultures 1 day after that first passage. As seen in the higher magnification on the right, the cells possess triangular or teardrop-shaped cell bodies that are distinct from one another and yet still closely packed.

Representative Morphology - Monolayer Neural Induction

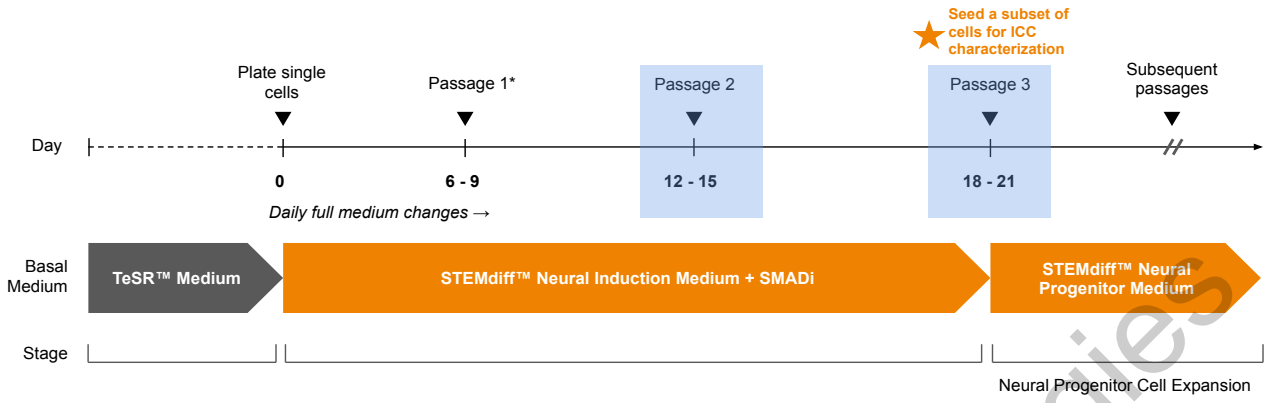
- 3 / 6 Days After Passage: Cells are proliferating and closely packed. In some cases, radial rosette-like patterns may form.



These images are both taken at the 10X objective magnification and demonstrate how that same culture looks after an additional few days. In the left image here, it can be seen that by 3 days after the first passage, the cells have proliferated to become more dense again. In some cases we can see these radial rosette-like patterns forming again. Recall however, that the appearance of these rosette patterns may differ between experiments and cell lines.

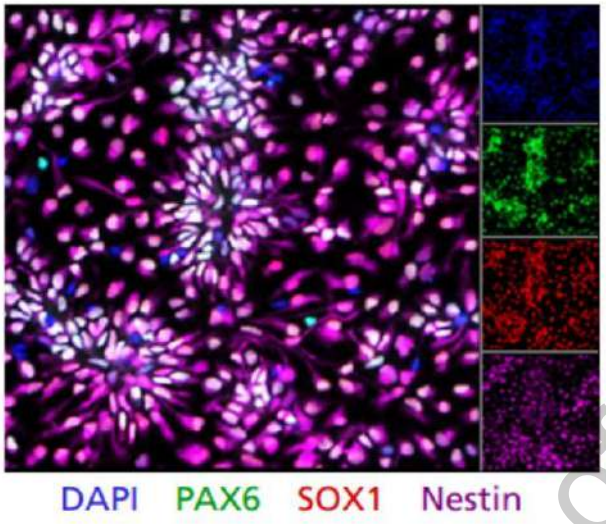
On the right, we can see this same culture 6 days after the first passage, and once again the cells have packed in very tightly as they were before, making it difficult to distinguish individual cells. This culture is considered ready for the second passage.

Neural Induction: Monolayer Protocol



Looking back at the protocol diagram here, you may see this second passage as well as the third passage highlighted in blue. So at approximately Day 12 -15 and then again at Day 18 - 21, the cells are passaged in the same way as was done for Passage 1. As noted earlier, the cells need to be cultured in the induction medium for the full three passages in order to be fully patterned to the neural fate. At that third passage, it is 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.

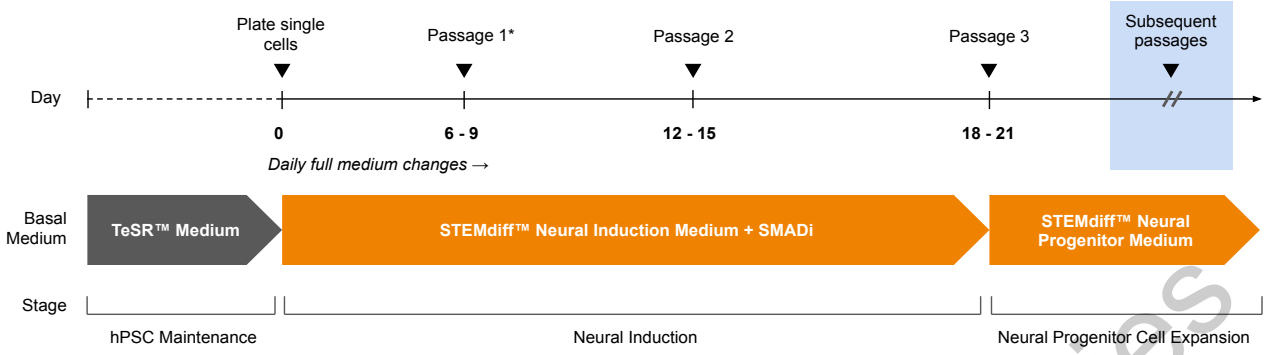
Neural Marker Expression: The Gold Standard



Successful neural induction:
>80-90% of cells expressing
NPC markers at Day 18-21

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 18-21 passaging step.

Neural Induction: Monolayer Protocol



Expected Expansion = 3- to 5-fold per passage

After this third 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 continue to 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 monolayer method for neural induction is simple and straightforward, and involves culturing hPSCs in STEMdiff™ SMADi Neural Induction Medium to promote a neural fate.
- A total of 3 passages in the induction medium are required before proceeding with NPC expansion or further differentiation.
- **Key steps of the monolayer neural induction protocol include:**
  - Starting with high-quality hPSCs that have had areas of spontaneous differentiation removed.
  - Ensuring that cells are seeded at a high density at each plating and passaging step.
  - Checking for positive expression of neural markers (>80-90% PAX6, SOX1, Nestin) at the end of the induction.

In summary, the monolayer method for neural induction is simple and straightforward, and involves culturing hPSCs in STEMdiff™ SMADi Neural Induction Medium to promote a neural fate. A total of 3 passages in the induction medium are required before proceeding with NPC expansion or further differentiation.

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