

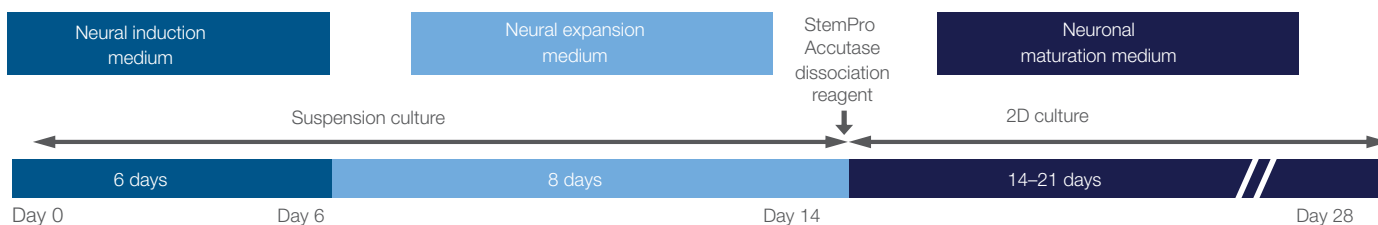
Neural differentiation from spheroids grown in StemScale PSC Suspension Medium

Introduction

Pluripotent stem cells (PSCs) enable the generation of a nearly unlimited pool of cells for downstream differentiation, disease modeling, drug discovery, and therapeutic applications. While two-dimensional (2D) expansion of PSCs and downstream differentiation is well established, the scale at which PSCs and subsequent PSC-derived cell types can be efficiently generated using traditional methods is limited without a significant increase in hands-on time, as well as a potential risk of contamination. To overcome these challenges, a method for expanding PSCs into three-dimensional (3D) spheroids in suspension using Gibco™ StemScale™ PSC Suspension Medium (Cat. No. A4965001) was developed for large-scale cell culture. Notably, StemScale medium enables efficient nucleation and size control of uniform spheroids yielding high cell

densities, which lowers overall cost, reduces hands-on time, and offers greater compatibility with closed systems compared to adherent culture media. To demonstrate the potential of expanded PSCs in suspension with downstream applications, we have developed protocols enabling efficient and scalable neural differentiation in 3D culture using reagents originally designed for monolayer culture (Figure 1). Key parameters and considerations have been identified and optimized for both PSC expansion and differentiation. Additionally, this approach has yielded wide-ranging benefits including faster differentiation and expansion steps, scalable expansion of neural stem cells (NSCs) and neural progenitor cells, and earlier onset of neuronal maturation and functional activity.

PSC Neural Induction Medium



PSC Dopaminergic Neuron Differentiation Kit

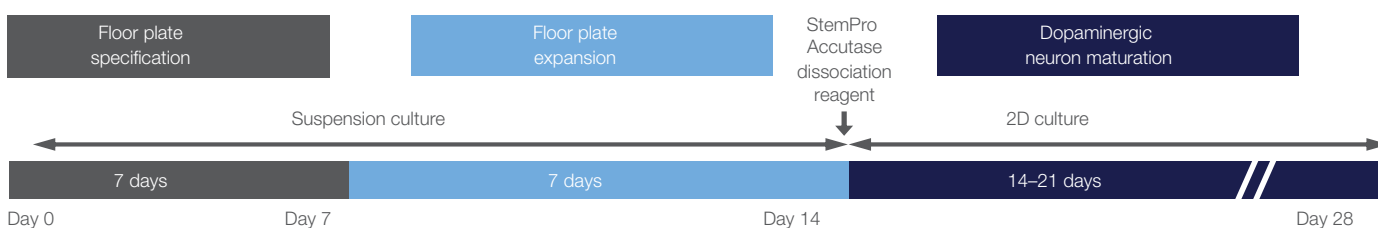


Figure 1. Schematic of neural differentiation processes from spheroids expanded in StemScale medium. Differentiation workflows are shown using Gibco™ PSC Neural Induction Medium and the Gibco™ PSC Dopaminergic Neuron Differentiation Kit.

Optimization of neural differentiation from spheroids grown in StemScale medium

PSCs expanded in StemScale medium grow as self-nucleating spheroids in suspension culture with agitation. For successful large-scale neural differentiation, spheroid cultures should ideally reach a uniform size. Unlike PSC medium developed for adherent culture, StemScale medium enables efficient nucleation of PSC spheroids, resulting in highly uniform spheroid cultures (Figure 2). In addition to uniformity, the size of PSC spheroids is a critical parameter that requires optimization for neural differentiation protocols. The size of PSC spheroids expanded in StemScale medium can be precisely controlled by the duration in culture as well as the speed of agitation.

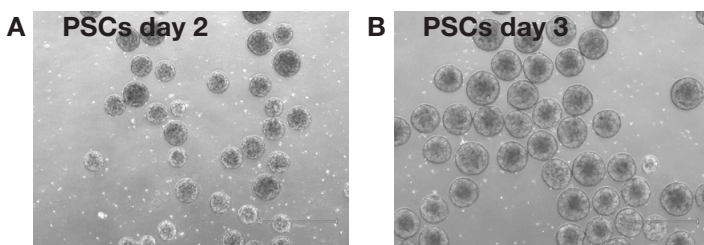


Figure 2. PSC spheroids formed in StemScale PSC Suspension Medium. Representative phase-contrast images of PSC spheroids at different times in culture are shown. **(A)** PSCs cultured in StemScale medium for 2 days (average diameter ~245 μm). **(B)** PSCs cultured in StemScale medium for 3 days (average diameter ~380 μm).

PSC spheroid size affects specification efficiency during neural differentiation

For optimization of the suspension culture protocol using the PSC Dopaminergic Neuron Differentiation Kit, we compared the impact of different spheroid sizes on differentiation efficiency. PSC spheroids with an average diameter of 200–250 μm resulted in highly efficient floor plate specification and high yield of TH-positive dopaminergic neurons (Figure 3). Larger sphere size at the start of differentiation resulted in low specification efficiency and low yield of dopaminergic neurons upon maturation. These results highlight the importance of sphere size optimization for neural differentiation protocols.

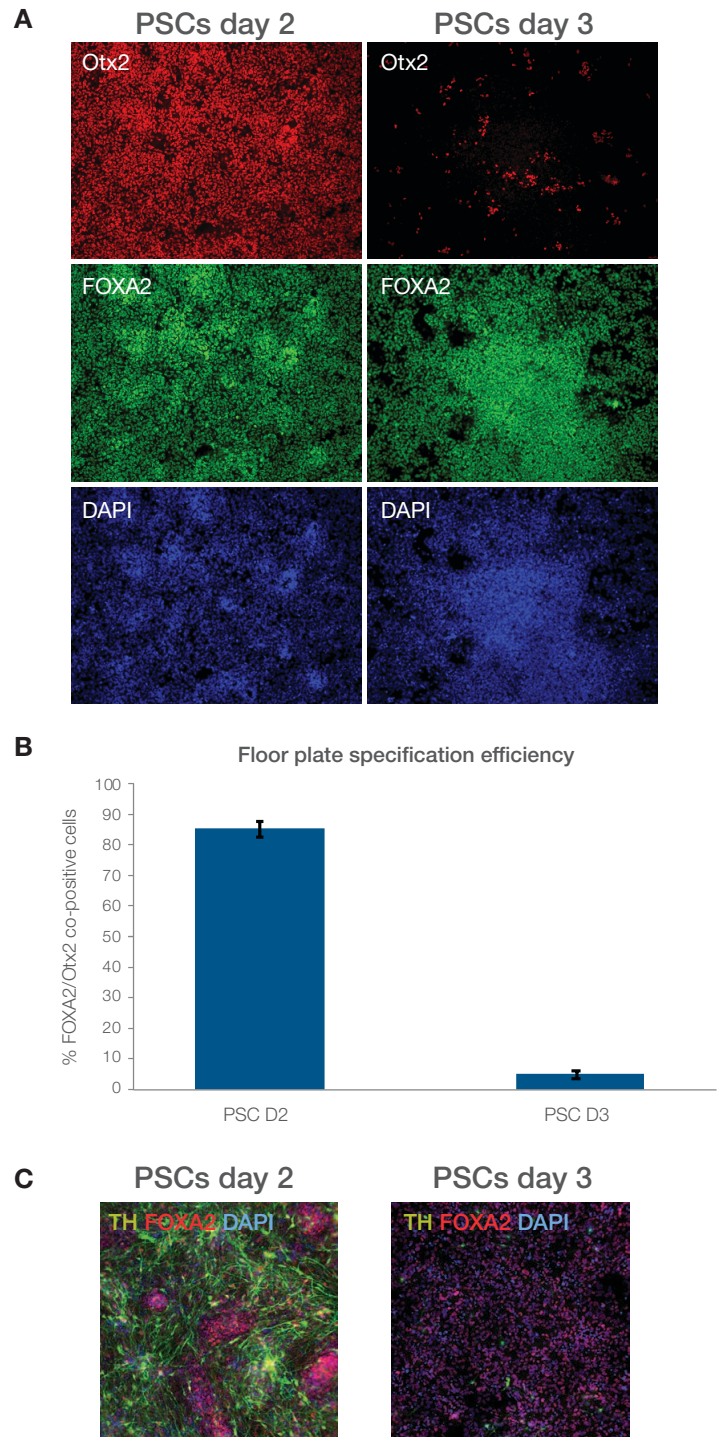


Figure 3. Impact of PSC spheroid size on neural differentiation in suspension culture using the PSC Dopaminergic Neuron Differentiation Kit. **(A)** After specification and expansion in suspension culture, cell aggregates were dissociated, plated in 2D, and stained for the floor plate-specific markers FOXA2 and Otx2. **(B)** Quantitative image analysis using the Thermo Scientific™ CellInsight™ CX5 High-Content Screening Platform showed over 85% FOXA2/Otx2 co-positive cells from the PSC day 2 cultures, compared to <5% from the PSC day 3 cultures. **(C)** Upon maturation of these cells in 2D culture, high levels of TH-positive cells (~30–40%) were observed in the PSC day 2 cultures compared to <2% in the PSC day 3 cultures.

Comparison of workflows for neural differentiation in suspension and monolayer culture

The benefits of performing neural differentiation in suspension culture vs. traditional monolayer culture include reduced protocol duration, steps, and hands-on time (Figure 4).

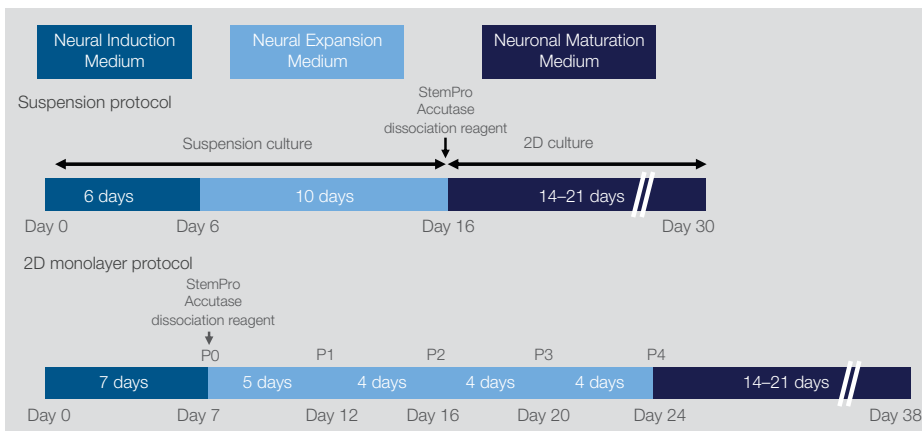


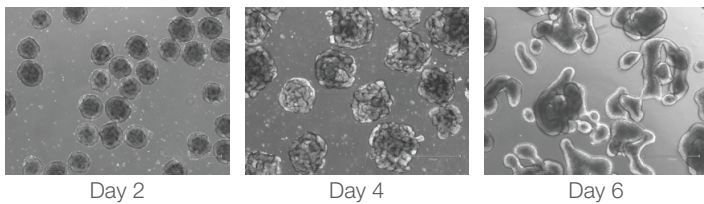
Figure 4. Comparison of protocols for PSC Neural Induction Medium: suspension vs. monolayer.

The suspension culture protocol can generate mature neurons in 30 days and requires one dissociation step, while the monolayer protocol takes 38 days and 5 dissociation steps.

Differentiation in a 3D environment offers significant improvements over standard 2D protocols

3D culture for PSC differentiation represents an environment more similar to *in vivo* conditions than monolayer differentiation. Cell aggregate morphology during 3D neural induction and expansion shows distinct epithelial-like aggregates throughout differentiation, indicating a more physiologically relevant environment (Figure 5).

3D neural induction (protocol day 0 to day 6)



3D neural expansion (protocol day 6 to day 16)

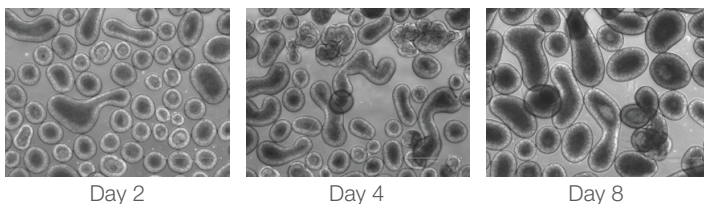


Figure 5. Cell aggregate morphology during neural induction and expansion in suspension culture. PSC spheroids cultured in StemScale medium were differentiated and expanded following the suspension culture protocol using Gibco™ PSC Neural Induction Medium. Representative phase-contrast images of cell aggregate morphology during induction and expansion are shown. During induction and expansion, cell aggregates become more structurally complex and form neural epithelial-like structures, indicating a more physiologically relevant environment compared to monolayer differentiation.

Neural differentiation and expansion in suspension culture results in NSC purity (>90% SOX1-positive cells) and positional marker expression similar to those of monolayer culture, while improving NSC yield more than 2-fold at day 12 of the respective protocols (Figure 6).

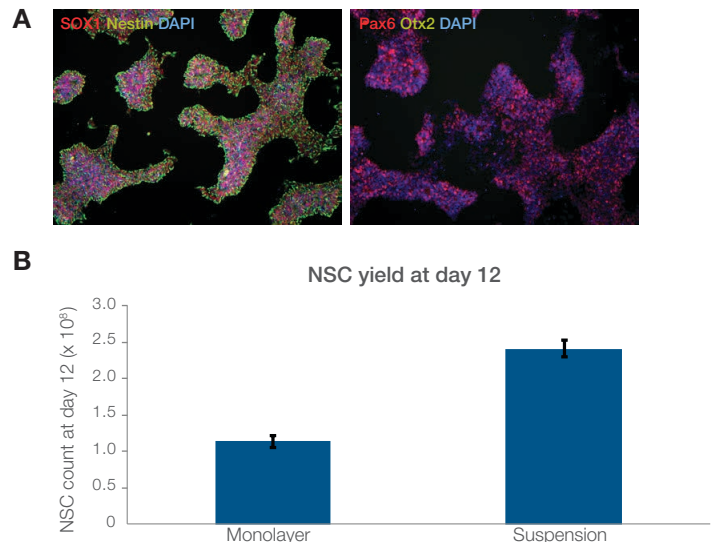


Figure 6. Equivalent purity and increased generation of NSCs in suspension culture. (A) Neural induction in suspension culture from spheroids cultured in StemScale medium yields highly pure (>90%) SOX1/nestin co-positive cells after 6 days of induction. Positional marker staining showed ~40% Pax6-positive cells and <5% Otx2-positive cells. Equivalent results are obtained from the monolayer neural induction method (not shown). (B) For each differentiation protocol, an equal number of PSCs (3.0×10^5) were initially seeded per well in 6-well plates. At day 12, total NSC yields were quantified. Monolayer culture yielded 1.14×10^8 NSCs, compared to 2.41×10^8 NSCs in suspension culture.

When comparing neuronal maturation of NSCs derived in 2D and 3D differentiation (Figure 7), we found that NSCs from suspension culture readily matured to neurons at an earlier time point (day 16 NSCs) than in monolayer culture (day 24 NSCs). This suggests that neural differentiation in suspension culture improves the NSC maturation process, resulting in highly pure neuronal populations in less time.

To further compare neurons derived through 2D and 3D differentiation, we examined the resulting functional activity of DA neurons as they matured over time. We found that DA neurons derived through suspension culture differentiation had significantly higher levels of functional activity as well as networked activity over time, compared to 2D-derived neurons (Figure 8).

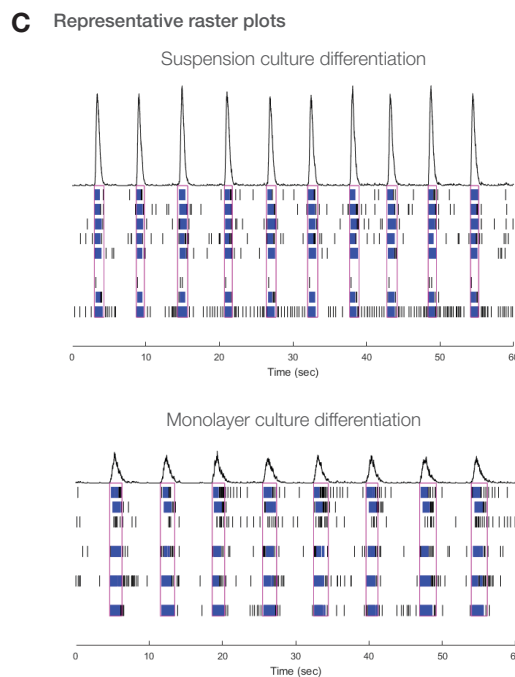
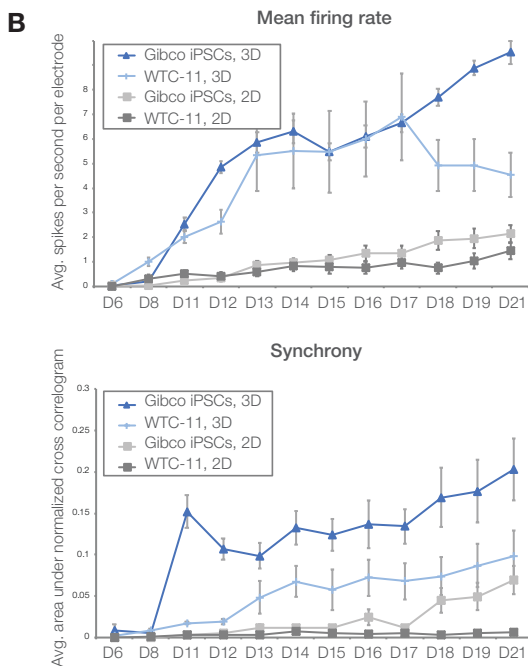
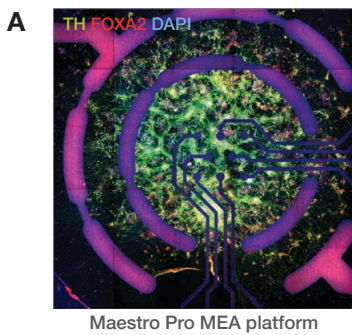


Figure 8. Improved functionality of neurons differentiated in suspension culture compared to the 2D method. (A) Image of dopaminergic neurons differentiated from floor plate progenitor cells that were differentiated in suspension culture and matured on the Maestro Pro™ microelectrode array (MEA) platform (Axion BioSystems). Cells were stained for TH, FOXA2, and nuclei (DAPI). (B) Two PSC lines, Gibco™ Human Episomal iPSCs and WTC-11, were differentiated in both monolayer and suspension culture with the PSC Dopaminergic Neuron Differentiation Kit. Floor plate progenitor cells were matured on an MEA plate, and spontaneous neuronal activity was measured over 23 days. Dopaminergic neurons generated from suspension culture demonstrated earlier onset of spontaneous network activity that was sustained for 10 days. (C) Representative raster plots of spontaneous activity of dopaminergic neurons differentiated in suspension and monolayer culture.

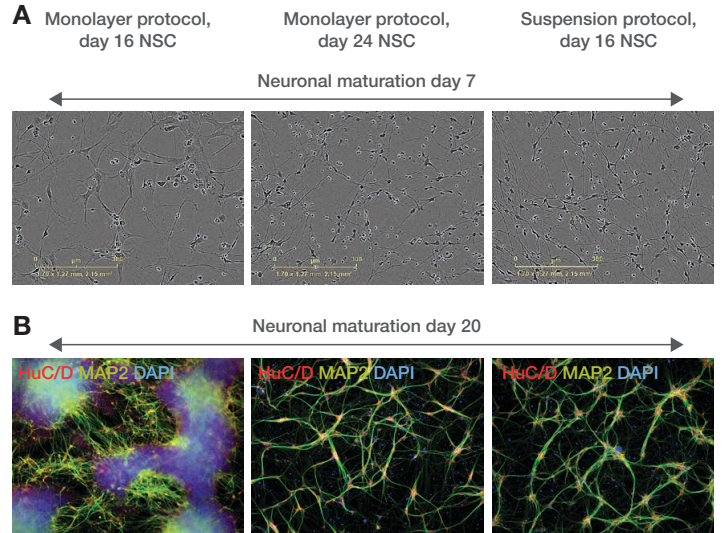


Figure 7. Suspension culture differentiation produces more mature NSCs. Neuronal maturation efficiency of NSCs derived from monolayer and suspension culture differentiation was compared. Phase contrast images at day 7 of neuronal maturation. (A) Phase contrast images at day 7 of neuronal maturation. Maturing NSCs from monolayer culture in early stages of expansion (day 16) show high levels of proliferative cells (left). Monolayer NSCs must be expanded to P4 (day 24) to obtain pure neuronal cultures upon maturation (middle). In contrast, suspension culture–derived NSCs require less time (day 16) to achieve highly pure neuronal cultures upon maturation. (B) After 20 days of neuronal maturation, cells were fixed and stained with neuronal markers HuC/D and MAP2 as well as nuclear marker DAPI. The presence of proliferative cells results in extensive cell clumping during maturation (left), while the later-stage monolayer NSCs (middle) and the suspension culture NSCs (right) result in highly pure neuronal cultures upon extended maturation.



Conclusions

StemScale PSC Suspension Medium enables efficient and scalable expansion of PSC spheroids. The applications highlighted here for neural differentiation in suspension culture demonstrate the compatibility of spheroids expanded in StemScale medium with differentiation reagents traditionally used for 2D culture. Compared to workflows using 2D culture alone, neural differentiation utilizing 3D culture offers multiple control points for optimizing differentiation, improved expansion and overall yields, and reduced time for generation of functionally mature neurons. Specific characteristics of PSCs cultured in StemScale medium, including efficient nucleation, spheroid uniformity, and precise control of spheroid size, enable the substantial benefits highlighted for neural differentiation.

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