Video Article

Rapid and Efficient Generation of Neurons from Human Pluripotent Stem Cells in a Multititre Plate Format

Miao Zhang[1], Hans R. Schöler[1,2], Boris Greber[1]

[1]Max Planck Institute for Molecular Biomedicine

[2]Medical Faculty, University of Münster

Correspondence to: Boris Greber at boris.greber@mpi-muenster.mpg.de

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Abstract

Existing protocols for the generation of neurons from human pluripotent stem cells (hPSCs) are often tedious in that they are multistep procedures involving the isolation and expansion of neural precursor cells, prior to terminal differentiation. In comparison to these time-consuming approaches, we have recently found that combined inhibition of three signaling pathways, TGFβ/SMAD2, BMP/SMAD1, and FGF/ERK, promotes rapid induction of neuroectoderm from hPSCs, followed by immediate differentiation into functional neurons. Here, we have adapted our procedure to a novel multititre plate format, to further enhance its reproducibility and to make it compatible with mid-throughput applications. It comprises four days of neuroectoderm formation in floating spheres (embryoid bodies), followed by a further four days of differentiation into neurons under adherent conditions. Most cells obtained with this protocol appear to be bipolar sensory neurons. Moreover, the procedure is highly efficient, does not require particular expert skills, and is based on a simple chemically defined medium with cost-efficient small molecules. Due to these features, the procedure may serve as a useful platform for further functional investigation as well as for cell-based screening approaches requiring human sensory neurons or neurons of any type.

Video Link

The video component of this article can be found at https://www.jove.com/video/4335/

Introduction

hPSCs, comprising embryo-derived human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), are pluripotent meaning that they can give rise to various cell lineages *in vitro* [1-2]. Numerous differentiated cell types have been derived from hESCs to date, supporting the notion that these cells present valuable tools for scientific research and cell-based screening approaches, and hold promise for cell-based therapeutics.

Neuronal differentiation protocols for hESCs are usually complex and time-consuming as they are often based on first inducing overall neural fate, followed by manual isolation of neural progenitors, and subsequent terminal differentiation into a given neuron type of interest[3-6]. In some regard, this complexity is not surprising and inevitable given that such state-of-the-art directed differentiation protocols tend to stepwise mimic early human development, which is in itself extremely complex. On the other hand, it may in part also reflect our lack of understanding of the underlying molecular processes, resulting in differentiation protocols that are still far from being fully optimized.

With regards to the conversion of undifferentiated hPSCs into neuroectoderm, Pera *et al*. found that suppression of BMP / SMAD1/5/8 signaling enhances neural induction of hESCs[7]. Moreover, inactivation of SMAD2/3 signaling has been shown to promote neuroectoderm formation[8]. Accordingly, combined inactivation of these two pathways tends to lead to more efficient neural induction of hPSCs[4,9]. More recently, we have shown that a third signaling pathway, FGF/ERK, serves as a strong repressor of the earliest steps of neuroectodermal commitment in hESCs. Conversely, simultaneous repression of all these three pathways lead to near-homogeneous conversion of hESCs into neuroectoderm within just four days[10]. Subsequently, highly efficient terminal differentiation into functional neurons was observed within eight days. The neurons obtained were likely to be sensory neurons of the peripheral nervous system, which may in part explain their rapid derivation[10]. Defects in sensory neuron maintenance is regarded a cause of certain human disorders such as familial dysautonomia[11]. For modeling such diseases based on hiPSC technology[12], for further functional characterization, as well as for applied purposes not requiring any particular type of neurons, this differentiation procedure may present a useful basis.

However, the differentiation strategy we originally employed involved formation of embryonic bodies (EBs) from clumps of hESC colonies[10], and this resulted in quite a degree of heterogeneity regarding EB sizes, and also appeared to compromise neuron formation efficiency in some cell

lines. Moreover, due to the heterogeneity in EB sizes, the ability to systematically test effects of additional growth factors or small molecules during and after neuron formation appeared to be somewhat confounded.

In this report, we adapted our previous procedure to a medium throughput-compatible, forced aggregation-based technique to generate EBs in a highly size-controlled manner, as also shown in other contexts[13]. Subsequently, the EBs generated in V-shaped wells of 96-well plates were transferred to U-shaped ultra-low attachment 96-well plates, to initiate neuroectoderm formation in suspension. Four days later, the EBs could be plated out giving rise to terminally differentiated neurons at high efficiency and homogeneity. Alternatively, day-4 EBs were dissociated into single cells and plated out as such, which resulted in low-density monolayers of human neurons. Coherent results were thus far obtained with two independently derived hESC lines, HuES6 and NCL3.

As a prerequisite, successful EB formation was strictly dependent on the use of a synthetic polymer, polyvinyl alcohol (PVA), together with ROCK inhibitor Y-27632 known to promote hESC survival[13-14]. As a result, homogeneity of the EBs formed in 96-V-plates was substantially enhanced compared to formation of EBs as mass cultures based on random splitting techniques. This system thus provides a significantly improved platform for neuroectoderm formation in suspension culture, followed by highly controlled neuron formation under adherent conditions.

Protocol

1. Preparation of Matrigel-coated Plates and Media

1. Preparation of Matrigel-coated plates Matrigel has been found to be a preferred substrate for the attachment of differentiating EBs and also promotes the efficient outgrowth of neurons from these[10].
    1. Thaw 10 ml of Matrigel overnight on ice.
    2. Next day, dilute the jelly-like Matrigel with two volumes of ice-cold DMEM/F-12 and prepare aliquots of 1 ml in prechilled 15 ml conical tubes which may be stored frozen at -20 °C.
    3. Decide upon a plate format for neuronal differentiation. For instance, as a starting point, we recommend to perform an initial round of differentiation in a 12-well format. Pre-cool four 12-well plates at 4 °C. Dissolve one aliquot of frozen Matrigel by adding 9 ml of pre-chilled DMEM/F-12 followed by pipetting up and down until all the prediluted Matrigel has thawed and dissolved.
    4. Then, transfer the contents into a new 50 ml tube containing 15 ml of DMEM/F-12 on ice (final Matrigel dilution: 1:75). Then, add 0.5 ml of diluted Matrigel to each well of the pre-cooled 12-well plates. Wrap plates with Parafilm and let stand at RT overnight.
    5. Next day, transfer plates to 4 °C. Coated plates can be stored for several weeks before use.

2. Media

EB formation medium (~15 ml needed to perform differentiation in one 96-well plate - **see Table 1** and scheme in **Figure 2**):
DMEM/F-12 with 0.4% (PVA)
1x N2
1x B27
0.05% BSA
20 ng/ml FGF2
10 µM Y-27632
1x PenStrepGln

Differentiation medium (~30 ml needed to perform differentiation in one 96-well plate, followed by neuron formation in one Matrigel-coated 12-well plate, **see Table 1**):
DMEM/F-12
1x N2
1x B27
0.05% BSA
0.5 µM PD0325901
15 µM SB431542
0.5 µM Dorsomorphin
1x PenStrepGln

2. Forced EB Formation

1. Grow hPSCs under your preferred feeder-free conditions. We use MEF-conditioned medium on Matrigel-coated 6-well plates[15]. However, other culture systems such as homemade or commercial defined media should also work. At the time of starting a differentiation experiment, hPSCs should be sub-confluent and actively growing.
2. Mechanically remove spontaneously differentiated colonies using a sterile plastic pipette tip under a stereo microscope.
3. Wash remaining undifferentiated colonies using PBS, and digest to single cells using Accutase containing 1X Y-27632. Pellet single cells by centrifugation at 200 x g for 2 min, resuspend in a small volume of EB formation medium and determine cell titre using a hematocytometer.
4. Add an aliquot of cells to the remaining EB formation medium to result in a titre between 40,000 and 80,000 cells per ml.
5. Using a multichannel pipette, transfer 100 µl per well to a 96V-bottom plate, resulting in 4,000 to 8,000 cells per well. Spin 96-well plate in a swing-out plate centrifuge at 400 x g for 1 min to collect cells at the bottom of the wells, and transfer to cell culture incubator. EBs will form overnight, as shown in **Figure 1**.

3. Neuroectoderm Induction

1. Next day (day 0), collect EBs from 96V plate under a stereo microscope and transfer in a small volume into a 3.5 cm bacterial dish containing 2 ml of differentiation medium. Gently agitate the dish for several seconds to wash the EBs.
2. Add 100 µl of differentiation medium per well of an ultra-low-attachment U-bottom 96-well plate. Under a stereomicroscope, transfer EBs in small volumes from the washing dish into the 96U-wells (one EB per well). Place 96U plate into cell culture incubator for 4 days to allow neuroectoderm formation.

4. Neuron Formation

1. On day 4, prepare a washing dish as in step 3.1 and additionally replace DMEM/F-12 in a pre-warmed Matrigel-coated 12-well plate by differentiation medium (1 ml per well).
2. Plating of EBs
    1. Collect EBs from 96U plate, wash these as above, and carefully seed them one by one into wells of the Matrigel-coated 12-well plate (about 8 EBs per well): EBs should be equally distributed within the wells to allow undisturbed outgrowth of neurons over the next days (see "day 5" image in **Figure 2**).
    2. Before transfer of the 12-well plate back into the incubator, allow EBs to loosely attach for around 10 min at RT. Then, carefully transfer 12-well plate to cell culture incubator. Neurons will grow out from the plated EBs in a radial manner within the next 4 days, as shown in **Figure 2** ("day 8" image on the right).

5. Neuron Formation as Monolayers

1. As an alternative to the steps of point 4), at day 4 of the procedure, collect EBs into a 3.5 cm bacterial dish containing 2 ml of differentiation medium, then transfer EBs in a small volume to a PBS-containing dish, and then into another dish containing Accutase.
2. Let digest to single cells, centrifuge at 200 x g for 2 min, resuspend in a small volume of differentiation medium and determine cell titre using a hematocytometer. Adjust titre to 1-2X10^6 cells per ml using differentiation medium (without Y-27632).
3. Plate cells out in pre-warmed Matrigel-coated 12-well plate (1 ml per well), and transfer to cell culture incubator. Neuron formation as monolayers was observed between days 7 to 8 (**Figure 3C**). It should be noted, however, that this monolayer variant of the procedure is not fully optimized with regards to cell survival and most suitable substrate.

Representative Results

In our previous report, in line with many others, EBs from hPSCs could be generated simply by replating aggregates upon routine splitting of hPSCs into low-attachment dishes[10]. This usually results in a wide distribution of resulting EB sizes (**Figure 1C**, top). Another problem resulting from this is that EBs maintained in suspension tend to aggregate with one another, leading to an even higher heterogeneity of EB sizes. To circumvent these problems, we therefore attempted to generate EBs by plating single hPSCs into low-attachment wells of multititre plates (**Figure 1A**). This resulted in no EB formation and cell death (**Figure 1B**, bottom right). Applying a known survival molecule for hPSCs, Y-27632, gave similar results. Likewise, applying polyvinyl alcohol (PVA) that has previously been shown to enhance EB formation of hESCs[16], tended to "gather" the hPSCs at the bottom of the 96V-wells but did not lead to EB formation. However, if combining Y-27632 with PVA supplementation, we were able to readily generate EBs from single-cell suspensions of hESCs, under chemically defined conditions (**Figure 1B**). Conveniently, EB sizes in this multititre plate format could be tightly controlled by seeding different numbers of cells per well, in contrast to using conventional techniques (**Figure 1C**, bottom).

Upon further incubation in the 96V plates, however, the EBs tended to stick to the bottom of the wells. Therefore, on the day after EB formation, it was required to transfer the EBs to ultra-low attachment 96U plates (**Figure 1A**). Neuroectoderm is then induced in this format using a small molecule cocktail consisting of PD0325901 (FGF/ERK inhibitor), SB431542 (TGFβ/SMAD2 inhibitor), and dorsomorphin (BMP/SMAD1 inhibitor), as described[10]. Following neuroectoderm formation using this small molecule cocktail, the EBs can be plated out to give rise to neurons on any format, provided that surfaces are coated with Matrigel; (**Figure 2**). By initiating EB formation with different numbers of cells (**Figure 1C**, bottom), we found that EBs generated from around 8000 cells tend to yield the best neuronal differentiation capacity. This is characterized by pronounced neuronal outgrowths from the plated EBs, reduced necrotic EB centers after plating, and high expression of sensory and pan-neuronal markers such as BRN3A and β-III Tubulin (**Figures 2 and 3A,B**). The overall neuronal differentiation efficiencies appear to be comparable to the original procedure (~70%) (**Figure 3**), which was applicable both to hESCs and hiPSCs[10]. We have thus far employed two independent hESC lines, HuES6 and NCL3, in the multiwell procedure, whereas cell line-dependent variability in differentiation efficiencies can generally not be ruled out.

Several applications of hPSC-derived neurons will require monolayers of differentiated cells instead of plated EBs. We therefore also investigated whether putative neuroectodermal cell spheres at day 4 of the protocol (**Figure 2**, middle) could be dissociated into single cells using Accutase; to then be plated out as monolayers. Indeed, upon plating out single cells of day-4 neuroectodermal cells, pronounced neuronal differentiation of these replated cells is obtained at efficiencies of approximately 60% (**Figure 3C**).

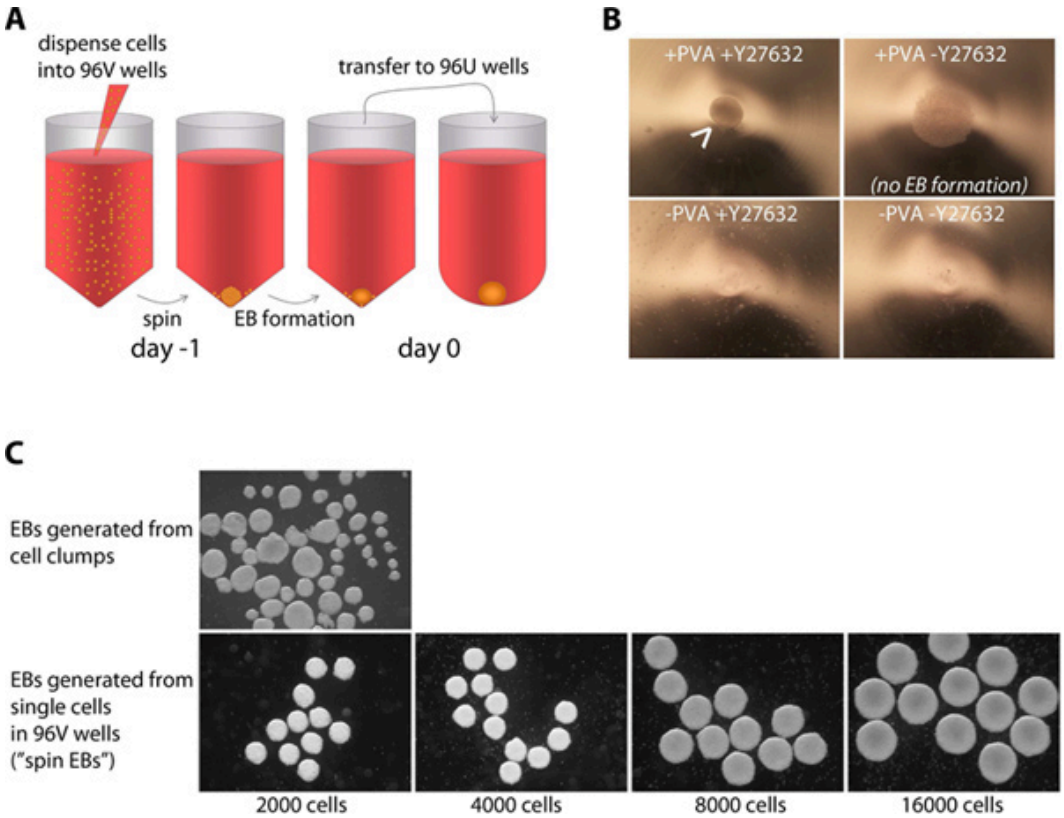

Figure 1. EB formation procedure. A) Schematic of the EB formation procedure. One day before initiation of differentiation (d-1), a suspension of hESCs containing several thousand cells per 100 µl is seeded into 96V wells, followed by centrifugation. EBs formed overnight are then transferred into ultra-low attachment 96U wells for further treatment. **B)** Testing the effects of PVA and Y-27632 on EB formation. EB formation could only be observed in medium containing both PVA and Y-27632 (see arrow head). **C)** Top: EBs formed by conventional means are usually heterogeneous in size. Bottom: EBs formed with "spin EB" technique depicted in **A.** By using different numbers of input cells, EB sizes can be tightly controlled and were highly uniform. Click here to view larger figure.

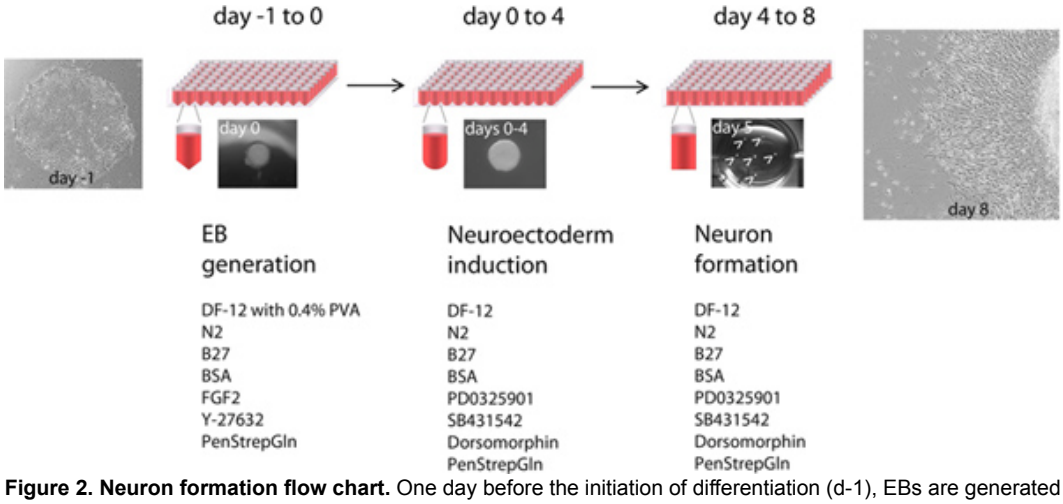

Figure 2. Neuron formation flow chart. One day before the initiation of differentiation (d-1), EBs are generated in 96V plates using the indicated EB formation medium. Upon transfer of the EBs into 96U plates, neural induction is initiated using differentiation medium. 4 days later, EBs are plated out in Matrigel-coated wells of a desired multiwell format. Representative morphologies are shown for each step. The picture on the right shows typical neuronal outgrowths from plated EBs - the high cell density area on the very right is part of the plated EB center from which the neurons tend to grow out in a radial manner. Alternatively, on day 4, EBs can be dissociated into single cells and plated out for terminal differentiation as monolayers (text and **Figure 3C** for details). Click here to view larger figure.

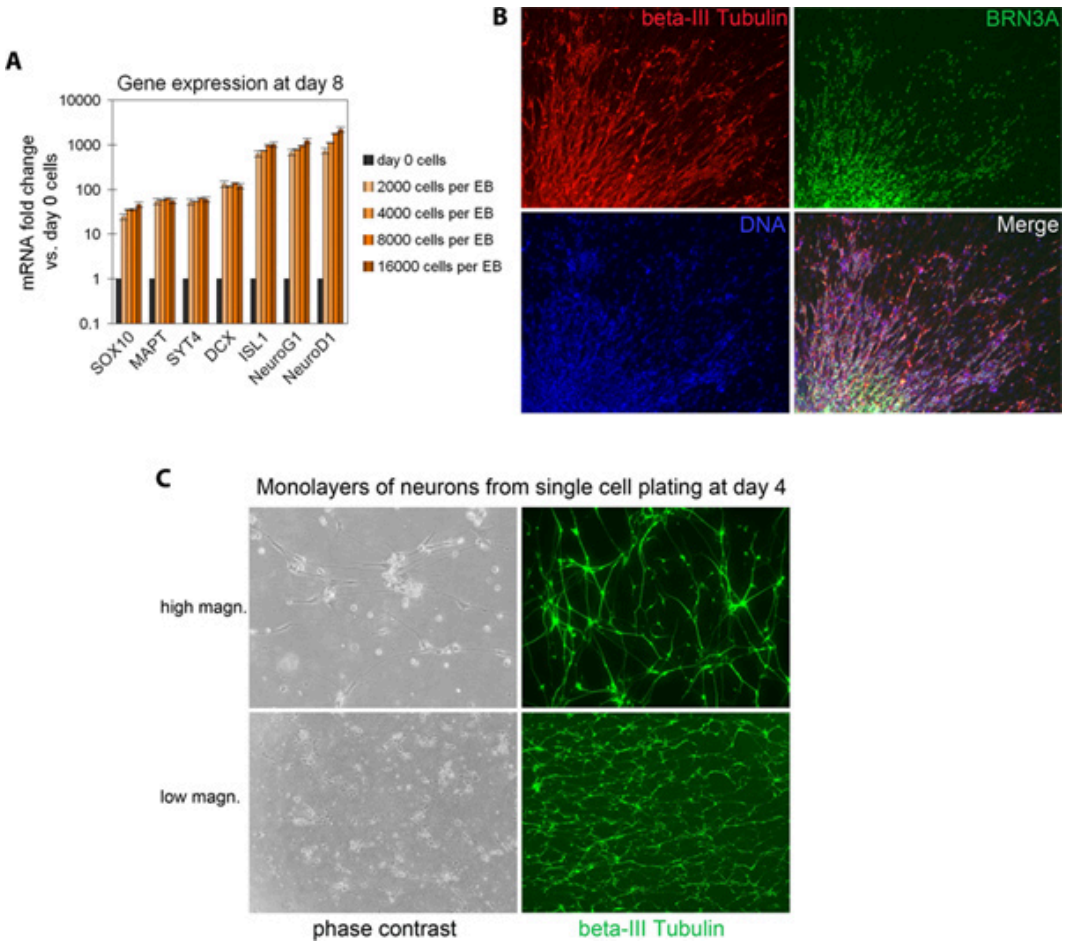

Figure 3. Characterization of neurons. A) Real-time RT-PCR characterization of day 8 bulk neuronal cultures. Note that in differentiated samples neural crest and pan-neuronal marker genes were strongly upregulated as compared to undifferentiated control cells. Although samples initiated with 16,000 cells showed the highest neuronal expression levels (note the logarithmic scaling), EBs between 4,000 to 8,000 cells proved most useful based on morphological criteria. **B)** Most cells growing out from the plated EBs are neurons staining positive for beta-III Tubulin (red) and the sensory neuron marker BRN3A (green). **C)** Neurons formed after single cell plating instead of plating EBs. On day 4 of the protocol (**Figure 2**), floating EBs were dissociated using Accutase and seeded out as single cells to then terminally differentiate as monolayers. As shown on the left, cells with typical neuronal morphology that stained positive for beta-III Tubulin were readily obtained, whereas cell survival appeared to be somewhat decreased as compared to EB plating. Right: Immunocytochemistry-based quantification of neuronal yield (n=3 representative sections analyzed +/- SD). Click here to view larger figure.

Name of gene	Fwd primer sequence	Rev primer sequence
ACTB (housekeeping gene)	TCAAGATCATTGCTCCTCCTGAG	ACATCTGCTGGAAGGTGGACA
RPL37A (housekeeping gene)	GTGGTTCCTGCATGAAGACAGTG	TTCTGATGGCGGACTTTACCG
DCX	AGGGCTTTCTTGGGTCAGAGG	GCTGCGAATCTTCAGCACTCA
ISL1	TTTATTGTCGGAAGACTTGCCACTT	TCAAAGACCACCGTACAACCTTTATCT
MAPT	TTACCCTGGGCACTGGCCTA	TCCTGCTCAACATGGCAAACTC
NEUROD1	GACTGATTGCACCAGCCCTTC	CGGACGGTTCGTGTTTGAAAG
NEUROG1	CAGAGACAAGGAGTGGGCTTCA	CTCCAGCCCTGTGCCTGAAT
SOX10	TCCTTGCCAGCTCCCTCTTC	CAAGGGTTAGGGCCTGAGCA
SYT4	GCCAATCTCACCACCCAAATG	CACACACCATGCTTCCTTCCA

Table 2. Primers used for RT-qPCR.

Discussion

Most differentiation protocols involving EB formation from hPSCs, including our previously published procedure[10], are based on manual or enzyme-assisted harvesting of hESCs as aggregates, which inevitably leads to heterogeneity in EB sizes. This fact leads to variability

in differentiation efficiency with some cell lines, thereby rendering systematic analyses difficult, in particular at a medium throughput scale. Furthermore, manual dissection is labor-intensive which by itself compromises scalability.

In contrast, the method described here is based on EB formation from single cells, which facilitates cell handling and minimizes variability between samples. Importantly, we show that overall differentiation capacity into neurons does not seem to be affected by initiating the protocol with spin EBs. Furthermore, keeping the individual EBs separated from each other by means of using 96-well suspension plates prevents them from aggregating with one another in suspension - which presents yet another drawback of the conventional procedure[10]. As a result, the amount of input hPSCs required to initiate a given experiment is low in that approximately one well of 6-well plate with subconfluent hPSC colonies is usually sufficient to initiate differentiation in two 96-well plates. Finally, the spin EB technique enables tight control over EB sizes. According to our observations, the expression levels of neuronal markers tend to increase to some extent with increasing sizes of plated EBs (**Figure 3A**). On the other hand, too large EBs tend to display necrotic centers after plating. Hence, as a compromise, we found EBs of 4,000 to 8,000 cells to be a suitable range.

Due to its high efficiency, simplicity, speed, and cost-effectiveness, we anticipate the described method to be a useful platform for functional studies, disease-modeling requiring human sensory neurons, or medium throughput cell-based pharmacological assays requiring neurons of any type. Regarding the latter application, compatibility with automated analysis such as high-content imaging would be desirable. This, however, may be compromised by the fact that only outgrowing neurons in the periphery of the plated EBs could be analyzed. To circumvent this obstacle, we also attempted to generate monolayers of neurons by dissociating EBs at day 4 of the protocol and plating out single cells. Indeed, the data in **Figure 3C** suggest that neurons formed at reasonable efficiency of approximately 60%. However, we observed a decrease in cell survival under these conditions, suggesting that this variation of the protocol may be further explored and optimized, for example by using a different attachment substrate. (The addition of Y-27632 upon plating out the day-4 cells in step 5.2 did promote cell survival after plating but it also appeared to interfere with subsequent neuronal differentiation and should therefore be omitted.) Furthermore, it will also be interesting to investigate whether other types of neurons could be generated with this platform, by varying the medium composition in the second stage of the protocol. Moreover, with regards to the transfer of the EBs from 96V into 96U-shaped wells at day 0, it would be desirable to devise a procedure based on multichannel pipetting, to ultimately achieve high-throughput compatibility of the described method.

Disclosures

No conflicts of interest declared.

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