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TITLE:

A Neurite Outgrowth Assay and Neurotoxicity Assessment with Human Neural Progenitor Cell-Derived Neurons

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KEYWORDS

Neurite Outgrowth Assay, Neurotoxicity Assessment, Human Neural Progenitor Cells, Screening, Small molecule compounds, Immunocytochemistry

SUMMARY

The presented protocol describes a method for a neurite outgrowth assay and neurotoxicity assessment of small molecule compounds.

ABSTRACT

Neurite outgrowth assays and neurotoxicity assessments are two major studies that can be performed using the presented method herein. This protocol provides reliable analysis of neuronal morphology together with quantitative measurements of modifications on neurite length and synaptic protein localization and abundance upon treatment with small molecule compounds.

Nowadays, cell lines are widely used in compound screening assays in neuroscience. While cell lines often differ genetically and phenotypically from their tissue origin, primary cells maintain

important markers and functions observed in vivo. Therefore, a neurite outgrowth assay could considerably benefit from using human neural progenitor cells (hNPCs) as the primary human cell model due to the translation potential and physiological relevance that these cells could offer.

In addition, neurotoxicity assessment can be performed using the method presented herein. Using this protocol, commercial chemical compounds could be tested to assess, distinguish and rank based on their potential developmental neurotoxicity effect.

INTRODUCTION

Neurite growth is a process fundamental to the formation of the neuronal network and nerve regeneration[1,2]. Following an injury, neurite outgrowth plays a key role in regeneration of the nervous system. Neurite outgrowth is also an important element of the extracellular signaling in inducing neuronal regenerative activities to enhance the outcomes for neurodegenerative disorders and neuronal injury[3–6].

By maintaining their differentiation potential in producing various neural lineages, human neural progenitor cells (hNPCs) could provide a model system for studies of central nervous system (CNS) function and development[7–9]. High translational potential and physiological relevance of hNPCs as a primary human cell model offer a considerable advantage in neurite outgrowth-related drug discovery screenings. However, the maintenance and scaling of the primary cell models for high-throughput assays could be time-consuming and labor-intensive[10–13].

In addition to the application of the presented method in neurite outgrowth studies, neurotoxicity assessment is another application using the hNPC-derived neurons. There are thousands of commercial chemical compounds that are either not examined or with poorly understood neurotoxicity potential. Therefore, more reliable and effective screening experiments to assess, distinguish, and rank compounds based on their potential to elicit developmental neurotoxicity is in high demand[14]. The increase in prevalence and incidence of neurological disorders along with the abundance of untested compounds in the environment necessitates the development of more trustworthy and efficient experiments to identify hazardous environmental compounds that may pose neurotoxicity[15].

The presented method herein can be utilized to screen for the ability of compounds to induce neurite outgrowth and neurotoxicity by taking advantage of the human neural progenitor cell-derived neurons, a cell model closely representing human biology.

PROTOCOL

Fetal specimens were received from the Birth Defects Research Laboratory at the University of Washington in Seattle through a tissue distribution program supported by the National Institute of Health (NIH). The Birth Defects Research Laboratory obtained appropriate written informed consent from the parents and the procurement of tissues was monitored by the Institutional Review Board of the University of Washington. All the work was performed with approval by the Human Subject Research Office at the University of Miami[8].

1. Isolation and culture of human neural progenitor cells (hNPCs)

1.1. Place the brain tissue in a 100 mm Petri dish and carefully remove the meninges using forceps.

1.2. Transfer the brain tissue to a 50 mL conical tube and wash it twice with 20 mL of PBS by gently inverting the tube.

1.3. Incubate the brain tissue in a new 50 mL conical tube by submerging the tissue in cell dissociation solution (see **Table of Materials**) and DNase I (10 U/mL) for 10 min at 37 °C.

1.4. Add 5 mL of neuronal cell culture medium (see **Table of Materials**) to the brain tissue containing tube and mechanically dissociate the neurospheres by triturating 20 to 30 times through a 1000 µL pipet tip to make a single-cell suspension.

1.5. Filter the cell suspension through a 70 µm cell strainer to remove cell clusters.

1.6. Seed the single-cell suspension in a vented T-25 flask provided with 5-10 mL of neuronal cell culture medium supplemented with components detailed in **Table 1**.

1.6.1. Sterilize the heparin solution by filtration through a 0.2 µm filter. The B-27 supplement without Vitamin A is a serum-free supplement for the cultivation of neural progenitors and stem cells, without inducing differentiation.

NOTEs: Human neural progenitor cells (hNPCs) are isolated from human fetal brain collected from aborted fetus. Following 7–10 days in culture, neural stem cells (NSCs) form free-floating neurosphere colonies, whereas other cell types remain in suspension as single cells or attach to the bottom of the flask. The isolated hNPCs can be cultured as neurospheres in suspension for several months[8,16,17].

2. Passaging the hNPCs

2.1. Collect the media containing the floating spheres, big and small neurospheres, and transfer them to a 50 mL conical tube.

NOTE: Due to unknown reasons, the timing for splitting the neurospheres is variable from 7 days up to 30 days. However, generally, neurospheres need to be passaged when they reach a diameter greater than 700-900 µm. This is when the center of the neurosphere starts to darken, which is considered as a sign of a high rate of cell death[18].

2.2. Spin the neurospheres down by centrifugation at 300-400 x *g* for 3 min.

2.3. Carefully aspirate the supernatant and then submerge the spheres in 500 µL of defrosted cell dissociation reagent (see **Table of Materials**).

2.3.1. Make aliquots of cell dissociation reagent by adding 500 µL per 1.5 mL microtubes and store at -20 °C. To avoid losing enzyme activity, thaw the cell dissociation reagent by holding at RT or warm for 5 min in a 37 °C water/bead bath.

2.4. Depending upon the density and size of the spheres, incubate the submerged spheres at 37 °C for 5-15 min.

2.5. Add 5-10 mL of pre-warmed culture media to the neurospheres containing 50 mL conical tube and centrifuge at 300-400 x *g* for 5 min to sediment the neurospheres.

2.6. Aspirate the supernatant and gently pipette up and down, using a 1000 µL pipette, in 2 mL of culture media until all the neurospheres are in a single cell suspension.

NOTE: The dissociation will become visible to the naked eye. Before dissociation, the neurospheres are in the form of spheres. After submerging in dissociation reagent and by pipetting up and down, they will become single cells.

2.6.1. Count the cells and plate the single cells, 2 to 3 million cells per T-25 flask in 10 mL of culture media.

2.7. Feed the cells every 3 days by replacing half the culture media.

2.7.1. Settle neurospheres by leaning the flask so that it is on its bottom corner. Hold the flask in the position for about 1-2 min until the neurospheres sediment. Then aspirate half the media gently by inserting the serological pipette in the media above the settled neurospheres. Dissociated cells can aggregate to form spheres after 2 to 3 days in culture[19].

3. Freezing the hNPCs

3.1. Prepare the cell freezing medium by adding DMSO to the culture media to a final concentration of 10% (v/v) or use commercially available cryopreservation medium for sensitive cell types (see **Table of Materials**).

3.2. Sort out big spheres by transferring the media into a 50 mL conical tube and letting the spheres settle by gravity. Then remove and transfer the big neurospheres into a new 50 mL conical tube for passaging using a 200 or 1000 µL pipet tip.

NOTE: Neurospheres with a diameter greater than 900 µm are considered big and the ones with a diameter smaller than 500 µm are considered small.

3.3. Spin the remaining neurospheres down by centrifugation at 300-400 x *g* for 3 min. Carefully remove the supernatant.

3.4. Resuspend up to 100 spheres in 1 mL of cryopreservation reagent and transfer it to a cryotube.

3.5. Store overnight at -80 °C in a cell freezing container (see **Table of Materials**) and move it to liquid nitrogen the next day for long-term storage.

NOTE: It is preferable to freeze small to medium-sized neurospheres (lower than 900 µm in diameter) and avoid freezing big-sized neurospheres (greater than 900 µm in diameter) or single cells. In order to reduce cell damage during thawing the sample, keep the cells dense by seeding the thawed neurospheres into a small T25 flask.

4. Differentiation and treatment of hNPCs

NOTE: To induce differentiation, neurospheres are disaggregated into single cells, counted and then seeded on coated plates for 5 days. Then differentiated cells are treated for 24 h with test compounds before immunostaining and fluorescence quantification.

4.1. Coating

4.1.1. Add 200 µL of poly-L-lysine (PLL) per well of 4-well glass chamber slides (140 µL per well of 8-well chamber slides).

4.1.2. Incubate for 1 h at room temperature (RT).

4.1.3. Wash 3x with PBS.

4.1.4. Let it dry at RT (for about 30 min).

4.1.5. Add 150 µL of laminin (50 µg/mL) per well of 4-well glass chamber slides (120 µL per well of 8-well chamber slides).

4.1.6. Incubate for 2 h at 37 °C.

4.1.7. Wash 3x with PBS.

NOTE: Coated chamber slides can be stored at 4 °C for 1 month.

4.2. Plating the cells

4.2.1. Count and plate 80,000 single cell neurospheres (neurospheres in a single cell suspension) per well of 4-well chamber slides (70,000 cells per well of 8-well chamber slide).

4.2.2. Add 500 µL of differentiation media per well of 4-well chamber slide (250 µL media per well of 8-well chamber slides).

4.2.2.1. To make the differentiation media first, add the following components in **Table 2** to a sterile, disposable container to make the neural inducing media (NIM).

4.2.2.2. Add the following ingredients in **Table 3** to 48.5 mL of NIM made in the previous step to make the differentiation media.

4.2.3. Incubate for 5 days at 37 °C.

4.2.4. After 5 days, treat the cells for 24 h by replacing half the media in each well with fresh media mixed with the desired concentration of test compounds, including appropriate controls.

5. Immunocytochemistry (ICC)

NOTE: Cells are fixed with 4% formaldehyde. Permeabilization and blocking is then performed to improve penetration and prevent nonspecific binding of antibodies. Cells are then incubated with primary antibodies overnight. Subsequently, cells are incubated with fluorescently labeled secondary antibodies. Finally, after using DAPI to stain the nucleus, chamber slides are mounted.

5.1. Fixation

5.1.1. Gently aspirate the media in each well.

5.1.2. Add 500 µL of 4% formaldehyde per well of 4-well chamber slides (250 µL per well of 8-well chamber slides).

5.1.3. Incubate for 15 min at RT.

5.1.4. Gently wash 2x with 500 µL of PBS.

NOTE: After fixation, by leaving 1 mL of PBS in each well, culture slides can be stored at 4 °C for up to 3 months.

5.2. Cell permeabilization and blocking

5.2.1. Add the following components in **Table 4** to a sterile, disposable container to make the antibody (Ab) buffer.

5.2.2. Mix the following ingredients in **Table 5** with the Ab buffer made in the previous step to make the cell permeabilization and blocking solution.

5.2.3. Add 500 µL per well of 4-well chamber slides and incubate for 1 h at RT.

NOTE: Ab buffer can be stored at 4 °C.

5.3. Staining

5.3.1. Wash 2x with 500 µL of PBS.

5.3.2. Add the diluted primary antibody, anti-β-tubulin III (1:200), and incubate overnight at 4 °C (200 µL per well of 4-well chamber slides). Dilute the primary antibody in PBS.

5.3.3. Wash 2x with PBS.

5.3.4. Add the diluted, in PBS, fluorescently labeled secondary antibody, Alexa Fluor 488 (1:500), and incubate for 2 h at RT in a place protected from light (250 µL per well of 4-well chamber slides)

5.3.5. Wash 2x with PBS.

5.3.6. Add the diluted, in PBS, DAPI (300 nM concentration) and incubate for 5 min at RT in a place protected from light (300 µL per well of 4-well chamber slides).

5.3.7. Wash 3x with PBS.

5.3.8. Mount the chamber slides using the following instructions.

5.3.8.1. Take apart the chamber slide by breaking the breakaway tabs and removing the gasket and base.

5.3.8.2. Add one drop of the mounting solution (see **Table of Materials**) per well of 4-well chamber slides. Then use a cover slide to cover the whole slide.

5.3.8.3. Using a tweezer and at an angle, place one side of the cover slip against the slide while making contact with the outer edge of the liquid drop.

5.3.8.4. Carefully tip the coverslip onto the mounting solution when lowering it into place. Avoid the creation of bubbles. Take a pipette tip and press it down on the cover slip. The bubbles will move to the side.

NOTE: Bubble formation is inevitable at times. If it occurs, image around them as long as there are a few.

5.3.8.5. Follow manufacturer's directions for curing time. Avoid using noncuring mounting solutions due to the difficulty in handling during imaging. Otherwise using an appropriate coverslip sealant on edges is required to prevent the coverslip from sliding during imaging.

5.3.8.6. To seal the coverslip, use nail polish and make a small line on the edge of the cover slip. Let the nail polish dry for about 2 min.

6. Image acquisition, neurite outgrowth and fluorescence intensity quantification

NOTE: Following staining, use a confocal microscope with a 20x objective and an image size of 1024 x 1024 pixels to acquire the images of the treated cells. Take image at least from two fields per biological replicate per condition. Then use Fiji image analysis software (ImageJ 1.51u) for quantification of the neurite length. Briefly, measure the length of the longest neurite for each neuron and after averaging the values per treatment, use student's t test for independent groups to compare the means between experimental groups and control group.

NOTE: Several commercial (Imaris, Volocity, Amira) and open source (ImajeJ, CellProfiler, Vaa3D, BioImageXD, Icy, KNIME) image processing programs are available. Among these programs, ImageJ has become the tool of choice for biological image analysis[20,21]. The ImageJ portal at *https://imagej.net/Introduction* is a useful source of information providing a thorough description of ImageJ's basic, and built-in functions including image processing, colocalization, deconvolution, registration, segmentation, tracking, and visualization.

6.1. Measuring neuronal outgrowth with Fiji image analysis software

6.1.1. As illustrated in **Figure 1**, open the image either by dragging and dropping it onto Fiji software or by selecting **File | Open**.

6.1.2. Select **Analyze | Tools | ROI manager**, and then right-click on the 5th icon in the toolbar **Straight** and switch to **freehand line**. Optionally, double-click on the same icon to change the line width to 10, and then trace the longest neurite, beginning near the cell body and extending to the tip of the neurite.

6.1.3. Press **Ctrl + T** & then **F** to add the measurement to the ROI Manager and highlight the measured neuron. Select all numbers in the **ROI**, click on **Measure**, select all calculated lengths, and copy/paste into a spreadsheet.

6.2. Measuring fluorescence intensity of labeled cells with Fiji image analysis software

6.2.1. As illustrated in **Figure 2**, after opening the image, click on the 4th icon in the toolbar **Freehand selections**. Draw the shape of the cell.

6.2.2. Select **Analyze | Set Measurements** and select for the following values: **Area**, **Integrated density**, **Mean grey value | Analyze | Measure** (a pop-up box with a stack of values opens).

6.2.3. Select a region next to the cell as background (size is not important) and then select **Analyze | Measure**. Select all measured data and copy/paste into a spreadsheet.

NOTE: Integrated Density (IntDen) is the item used for determining the fluorescent intensities. In this study fluorescent intensity of the target molecule is measured to initially analyze its distribution in the cell and subsequently quantifying the abundance of the target molecule under various treatments. Consequently, the effectiveness of treatments will be measured and compared with control.

7. Neurotoxicity assessment

NOTE: Cytotoxicity of test compounds are evaluated in 384-well plates (see **Table of Materials**) using a luminescent cell viability assay (see **Table of Materials**). The hNPCs are prepared following the same method, except slight modifications, described in the "Differentiation and Treatment of hNPCs" section. Subsequently, a luminescent signal generated by luminescent cell viability assay is measured utilizing a microplate reader. The luminescent signal is proportional to the cellular ATP concentration which itself is directly proportional to the number of viable cells present in each well.

7.1. Coating

7.1.1. Add 30 µL of poly-L-lysine (PLL) per well of 384-well plate.

7.1.2. Incubate for 1 h at RT.

7.1.3. Wash 2x with PBS.

7.1.4. Let it dry at RT (for about 30 min).

7.1.5. Add 30 µL of laminin (50 µg/mL) per well of 384-well plate.

7.1.6. Incubate for 2 h at 37 °C.

7.1.7. Wash 2x with PBS.

NOTE: Coated 384-well plates can be stored at 4 °C for 1 month.

7.2. Plating the cells

7.2.1. Count and plate 20,000 single cell neurospheres per well of 384-well plate in 25 µL of differentiation media.

7.2.2. Incubate for 5 days at 37 °C.

7.2.3. After 5 days, treat the cells for 24 h by test compounds prepared at 6x the final desired concentration in 5 µL volume (to make the final volume of 30 µL per well).

7.3. Cell viability assay

7.3.1. Add 30 µL of luminescent cell viability assay reagent per well of 384-well plate.

NOTE: Add a volume of luminescent cell viability assay reagent equal to the volume present in each well. Thaw the luminescent cell viability assay reagent and equilibrate to RT prior to use.

7.3.2. Shake on a plate shaker for 2 minutes (to mix and induce cell lysis).

7.3.3. Spin the mixture down by centrifugation at 300-400 x *g* for 30 s.

7.3.4. Incubate the 384-well plate for 10 min at RT in a place protected from light (to stabilize the luminescent signal).

7.3.5. Record luminescence with a microplate reader.

NOTE: Use appropriate controls for the viability assay including Velcade (at a final concentration of 10 µM) as positive and HBSS containing DMSO (with final concentration of 0.1% or 0.2%) as negative control.

REPRESENTATIVE RESULTS

The protocol presented in the manuscript has successfully been used in two recently published papers[22,23]. **Figure 3** demonstrates the use of hNPCs-derived neurons in examining the effect of HDAC inhibitors as epigenetic compounds on the extension of neurites as a marker for neurite outgrowth and subsequent neurogenic ability of small molecule compounds.

Furthermore, in **Figure 4** the neurotoxicity of tested compounds (HDAC inhibitors) is also assessed by simultaneously differentiating the hNPCs in 384-well plates, showing the potential of the presented cell model (hNPCs) for neurotoxicity assessment and the ability to scale up to test neurotoxicity for a higher number of compounds.

In another paper by Sartor et al. (**Figure 5**), measurement of the neuronal cell fluorescence intensity to quantify the abundance of H4K5ac, as a histone mark, after treating the neurons differentiated from hNPCs with epigenetic modifier compounds is successfully demonstrated[23]. Additionally, as illustrated in **Figure 6**, in an unpublished work, the protocol has been used to visualize a presynaptic protein, synaptophysin, to check for the possible synaptogenic effect of small molecule compounds.

Figure 1. **Step-by-step workflow illustrating an approach for measuring neuronal outgrowth with Fiji image analysis software.**

Figure 2. **Step-by-step workflow illustrating an approach for quantifying neuronal cells fluorescence intensity with Fiji image analysis software.**

Figure 3. Neurite outgrowth assay with small molecule epigenetic compounds. (**A**) Representative fluorescent images (20x magnification) for treatment of hNPCs-derived neurons with hydroxamic-based HDAC inhibitors. Human neural progenitor cells are differentiated for 5 days; then treated for 24 h with 0.1% DMSO (as control), and Trichostatin A, JNJ26481585, SB939, and PCI24781 (as test compounds). Then immunostaining is performed with neuronal-specific β-tubulin III antibody (green) to visualize neuronal processes and quantify neurite lengths and DAPI (blue) to visualize cell nuclei. (**B**) Statistical analysis of neurite length in various groups. As depicted, all test compounds can induce neurite outgrowth. Quantitative analysis of neurite length is done using ImageJ software. Error bars represent SEM; *** $p < 0.001$, **** $p < 0.0001$. This figure has been modified from Bagheri et al.[22].

Figure 4. Neurotoxicity assessment using luminescent cell viability assay. hNPCs are seeded and differentiated to neurons in the 384-well plate according to the same method used for neurite outgrowth assay. Thereafter the effect of test compounds on the viability of cells after 24 h of exposure is measured. As shown, there is no significant toxicity upon treatments. One-way ANOVA is used for data analysis and a p value < 0.05 is considered statistically significant. This figure has been modified from Bagheri et al.[22].

Figure 5. RGFP966 increases H4K5ac in neurons differentiated from human neural progenitor cells. (**A**) Representative immunofluorescence staining of β tubulin, DAPI, and H4K5ac following treatment with DMSO, RGFP966, or RGFP966 + JQ1 in hNPCs-derived neurons. (**B**) Quantification of fluorescence intensity of H4K5ac following treatment with DMSO, RGFP966, or RGFP966 + JQ1. Error bars represent SEM; **** $p < 0.0001$. This figure has been modified from Sartor et al.[23].

Figure 6. Representative immunofluorescence staining of Synaptophysin (red) to visualize synaptic terminals, β tubulin (green) to visualize neuronal processes, and DAPI (blue) to visualize cell nuclei following treatment with DMSO (as control), and SB939 (as test compound) in hNPCs-derived neurons.

DISCUSSION

This protocol is one of the few published papers describing the test for neurite length upon treatment with test compounds. Furthermore, we describe how to use hNPCs for a neurite outgrowth assay and neurotoxicity assessment. By utilizing this neurite outgrowth assay and neurotoxicity assessment on hNPCs-derived neurons, the neurogenic potential of a category of epigenetic small-molecule compounds, HDAC inhibitors, in inducing neurite outgrowth is demonstrated[22]. Furthermore, in another paper presented by Sartor et al., this protocol is used to quantify the fluorescence intensity of H4K5ac, a histone protein, upon treatment with several epigenetic modifier compounds[23].

Cognitive function relies on proper wiring and functional connections within neuronal circuits. Detailed and consistent quantification of various aspects of neuronal morphology as a phenotypic screening approach is essential to achieve reliable insight in the underlying pathways leading to brain disorders ranging from neurodevelopmental to psychiatric disorders. Phenotypic screening is considered a notably effective approach in exploring small-molecule compounds as putative

drug candidates to modulate a cellular phenotype. In this approach, instead of interrogating only a single target, all components and pathways of the cell are examined[24].

Neuronal morphology including shape, structure, and connectivity are considered to be key features of neuronal function. Genetic perturbations that alter the morphology of neurons or synaptic protein localization and level could notably contribute to the analysis of disease-causing mutations. Therefore, reliable methods are required to assess the impact of perturbagens on neuronal morphology[25].

The hNPCs, are isolated from the embryonic mammalian brain, which are subsequently cultured in vitro in the presence of mitogens. These cells are made up of neurospheres that are characterized as floating cellular aggregates comprised of neural progenitor cells and radial glial cells. The hNPCs provide a desirable model system for nervous system function and development by maintaining their differentiation potential in producing various neural lineages[7–9]. Neurite number, intensity, length, and width together with synaptic characteristics including pre- and post-synaptic proteins modifications are among neurite and synaptic changes that could be reliably and efficiently measured using the presented method herein.

Due to an increase in the prevalence of neurological disorders such as attention deficit hyperactivity disorder and autism, the neurotoxicity potential of environmental chemicals on children remains a public concern[15,26]. Therefore, the usability of hNPCs-derived neurons for reliable and efficient neurotoxicity assessment is also examined. As demonstrated in **Figure 4**, hNPCs could be adapted and scaled up for neurotoxicity assessment in 384-well plates.

Neurotoxicity assessment as an application for the introduced cell model is feasible by either differentiating the neurospheres into neurons before plating in 384-well plates or differentiating the cells while the neurospheres are plated in 384-well plates as single cells. In the latter approach, high precision pipetting skills are required for plating the exact number of neurospheres that are disaggregated into single cells and also for the following treatment procedures with differentiation-inducing medium. Considering the pipetting skills required, differentiation before plating is recommended to achieve more reproducible results. Moreover, bulk cell sorting by fluorescence activated cell sorting (FACS) could also potentially be used to accurately separate the required number of cells circumventing the need for high precision pipetting skills.

It is advised to start the neurite outgrowth assay by neurotoxicity assessment to avoid repeated neurite outgrowth assays with various doses of the same compound to reach for nontoxic concentration. Therefore, combining a neurotoxicity assessment by utilizing the luminescent cell viability assay against a dose-response curve of test compounds will result in finding the right dosage with minimum effort. The luminescent cell viability assay is a highly sensitive method to determine the number of viable cells in a cell culture. The method works on quantitation of the ATP present in culture as an indicator of cellular metabolism. The add, mix, and measure format of the assay provides a simple and rapid approach to check for cell viability and cytotoxicity of test compounds.

There are some limitations of the presented protocol. Due to availability and inherent variability, the use of physiologically relevant primary cells is limited[25]. hNPCs have a slow proliferation rate, which could limit its utilization. Manual large-scale analysis of fluorescent images of neurons is time-consuming and susceptible to experimenter bias. hNPCs could be considered as young neural cells, not representing age-related epigenetic modifications accumulating in cells throughout a person's life. As neurodegenerative disorders occur in older individuals, hNPCs may lack adequate physiological relevance to be an appropriate model for late onset conditions.

Even though animal models and cell lines have been valuable in deciphering molecular mechanisms underlying diseases, they have shown limitations in translating findings into human therapeutics[27–30]. Human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs) alongside hNPCs are considered as better in vitro cell models, recapitulating human physiology. Therefore hiPSCs- and hESCs- derived neurons can also potentially be used as two alternative cell sources for neurite outgrowth assay and neurotoxicity assessment, utilizing the protocol presented herein[1,31].

In conclusion, high translational potential and physiological relevance of hNPCs as primary human cell model provides a valuable recourse in neurite outgrowth-related drug discovery screenings and neurotoxicity assessment outweighing the limitations mentioned earlier.

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All authors indicate no potential conflicts of interest.

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Figure 1

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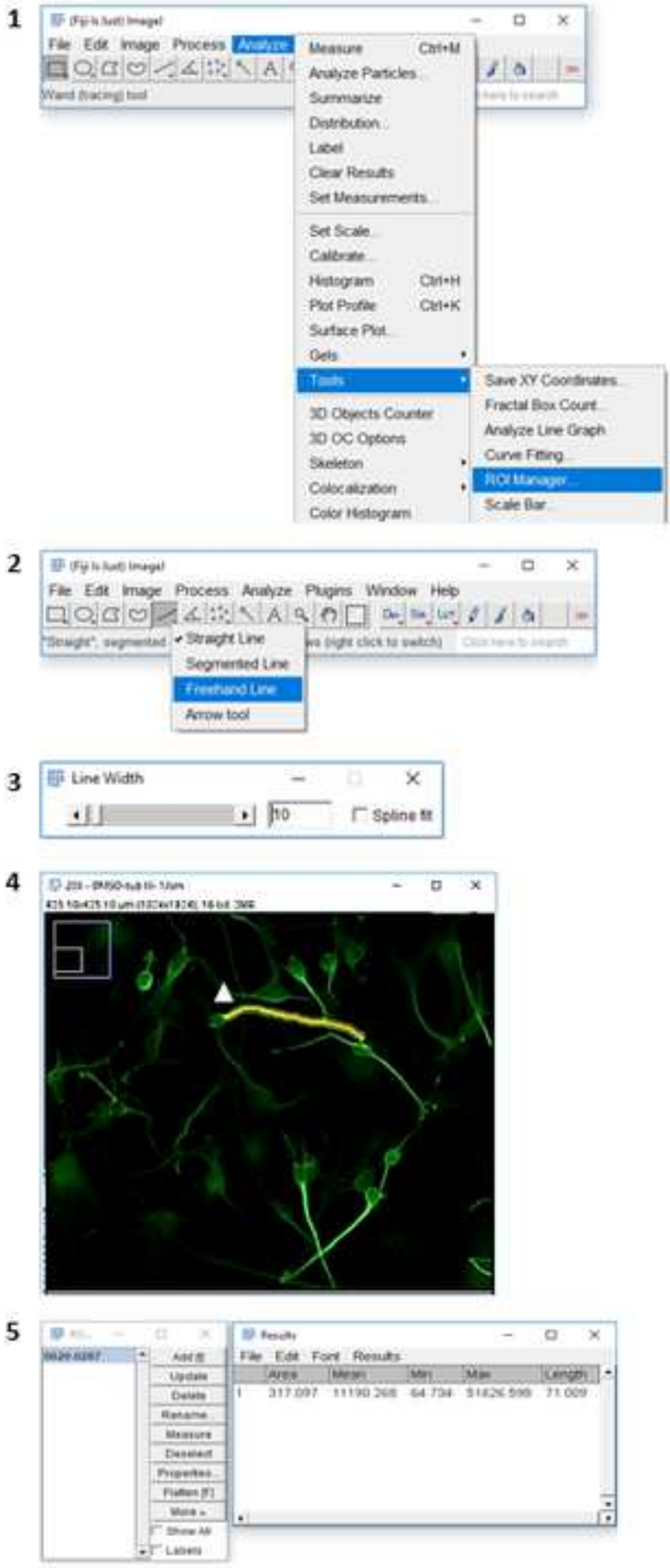

Figure 2

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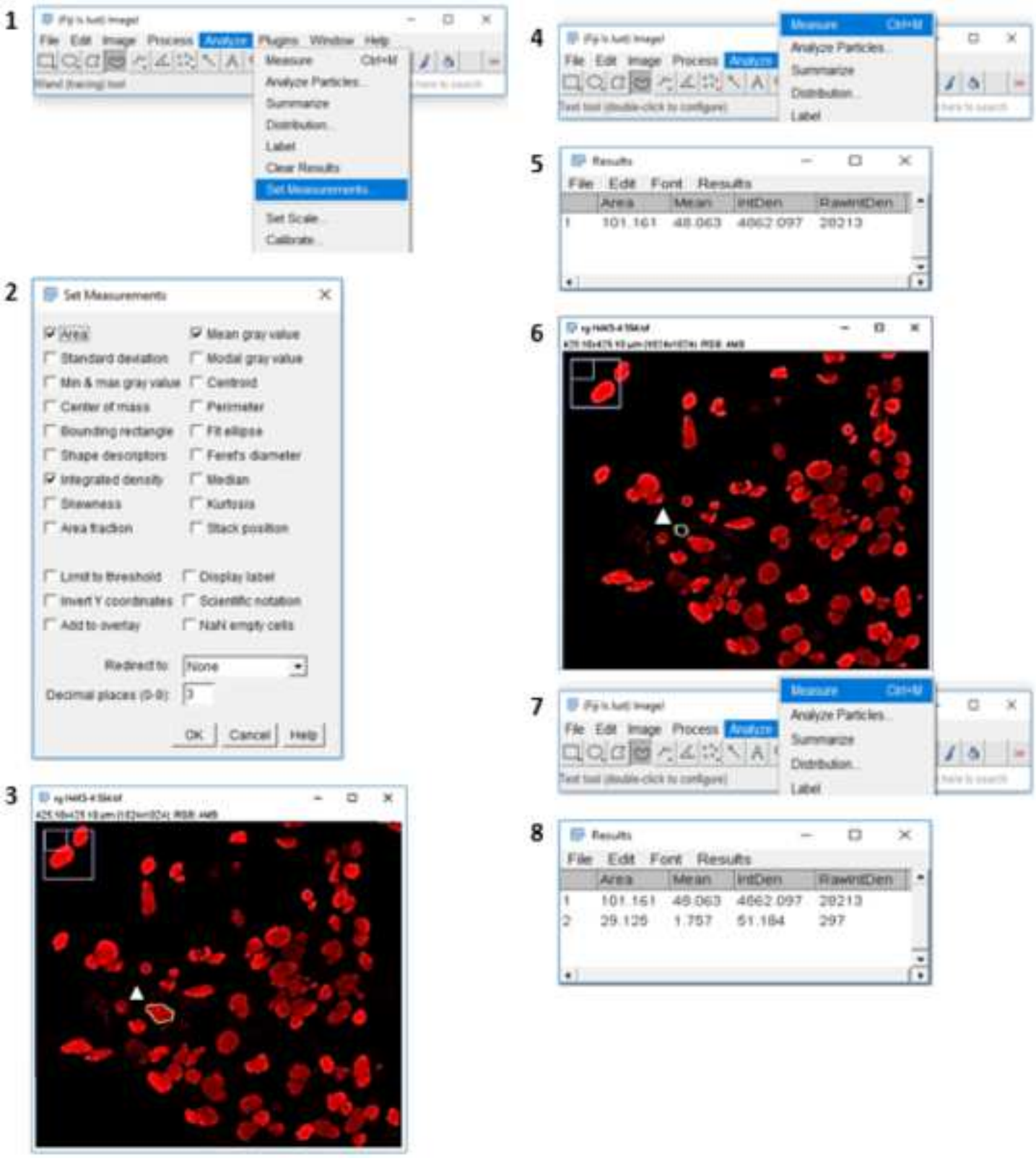

Figure 3

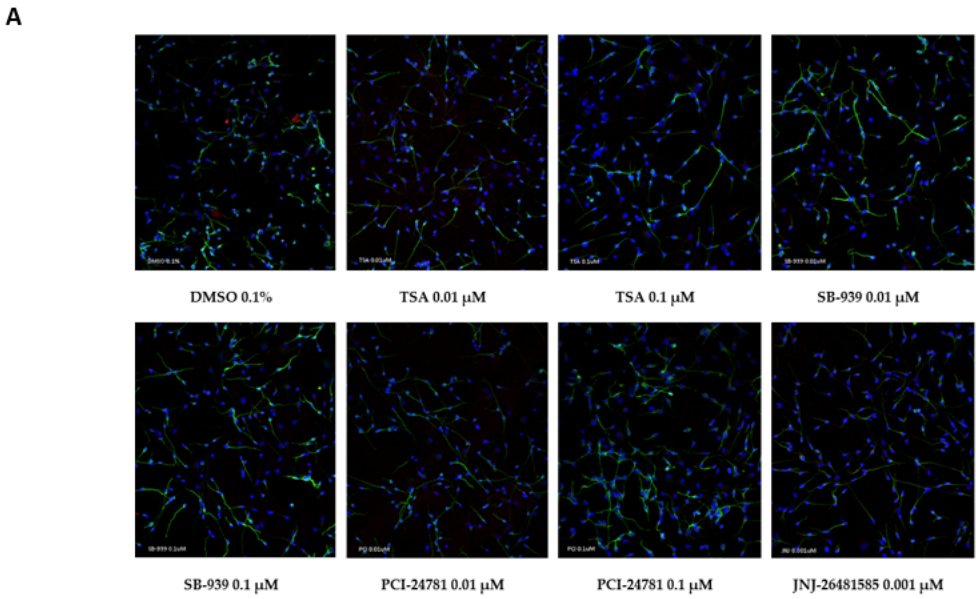

Figure 4

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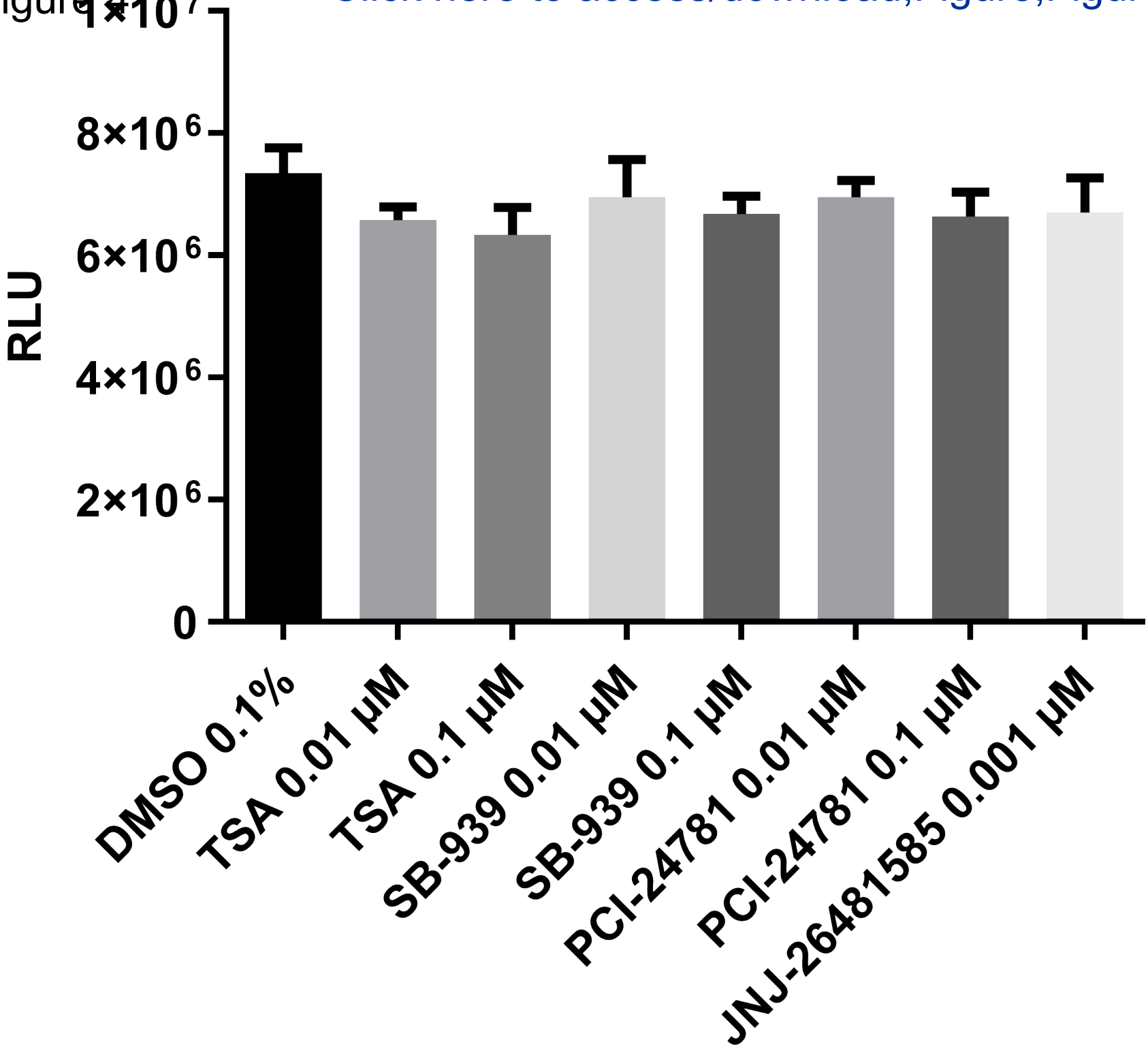

Figure 5

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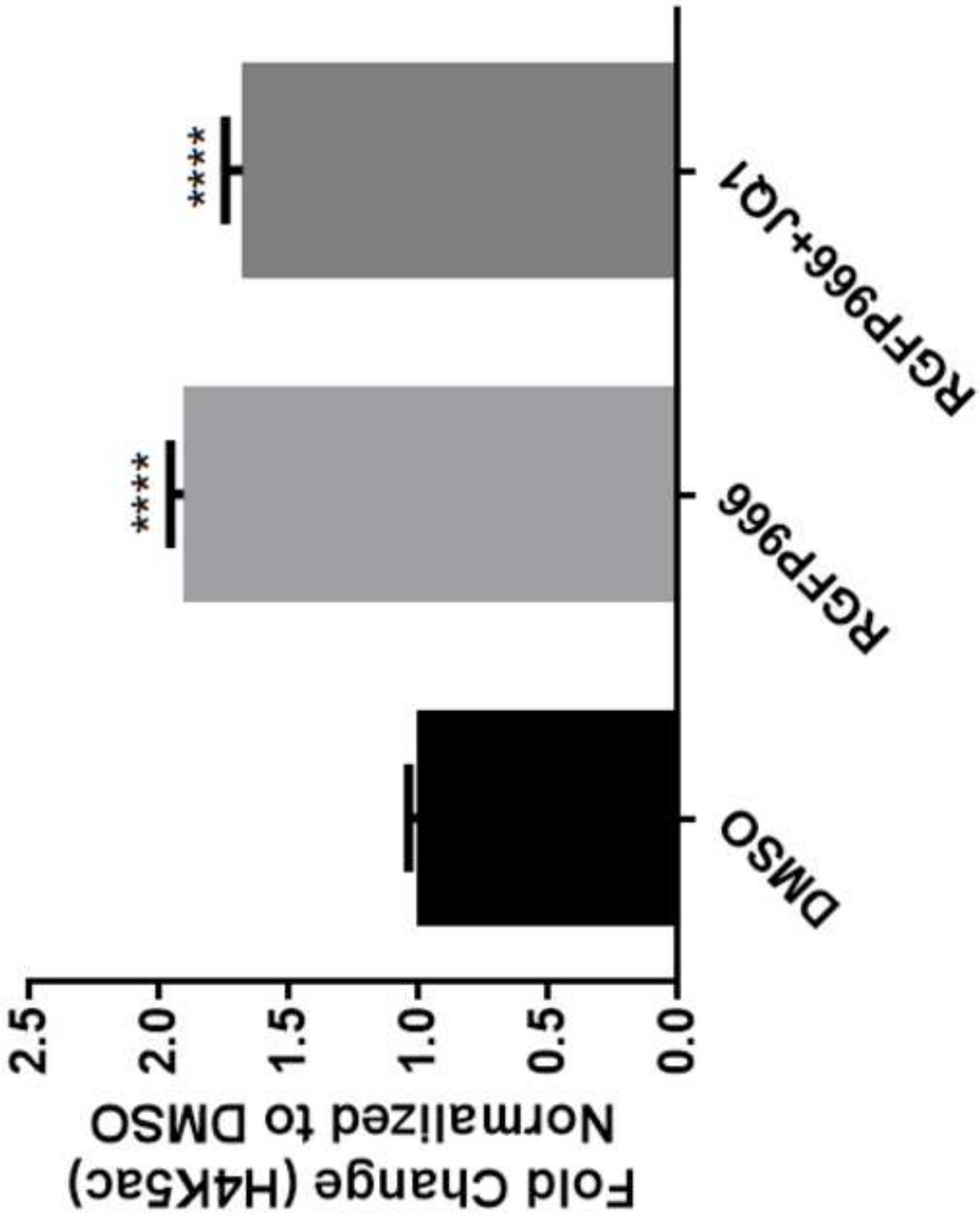

Figure 6

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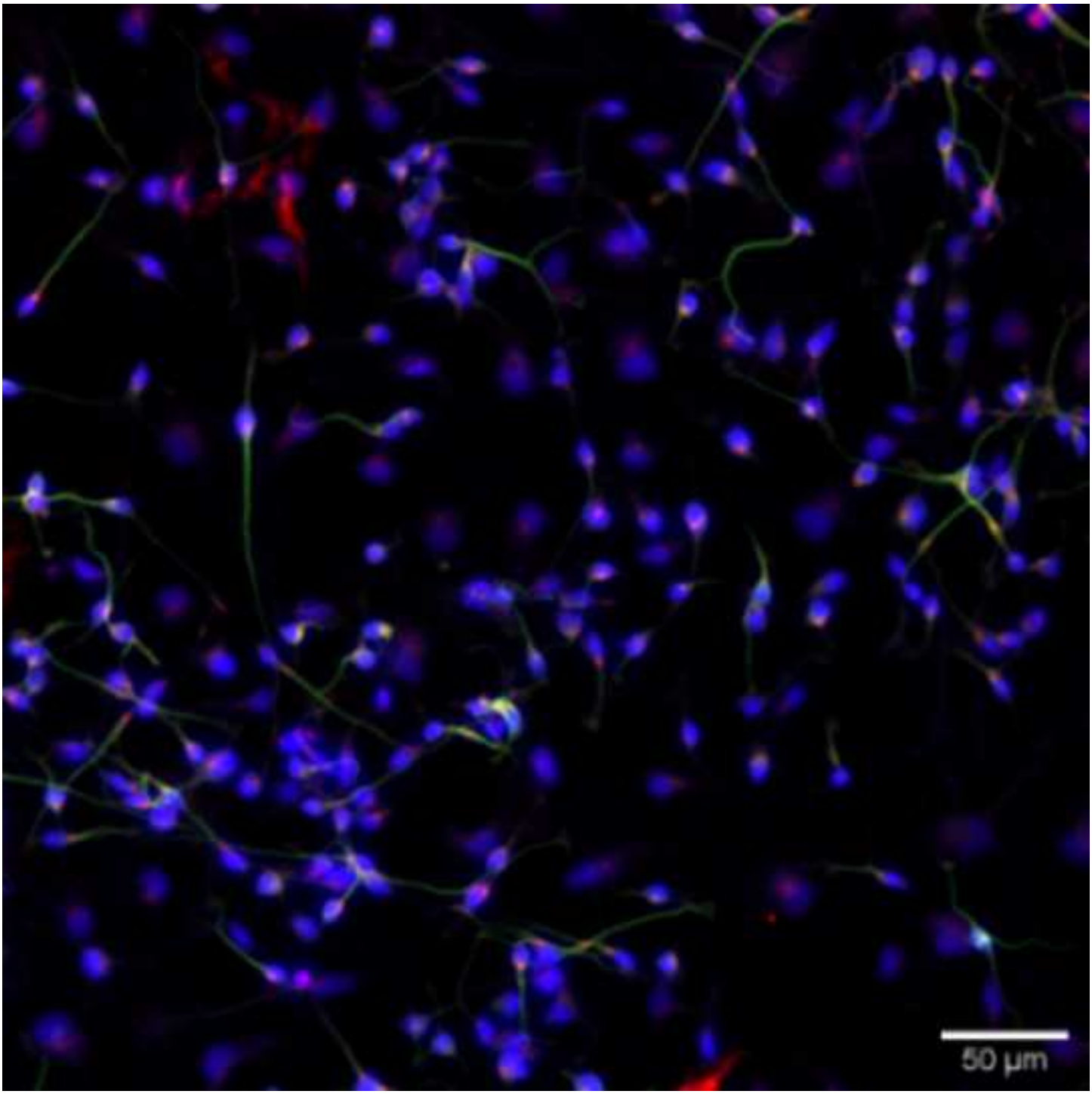

Table 1. Components required for making 100 mL of culture media

Amount	Component
100 µL	EGF (20 ng/mL)
100 µL	FGF (10 ng/mL)
2 mL	B-27, Minus vitamin A (50X)
1 mL	L-alanyl-L-glutamine (100X) (see table of materials)
4 µL	Heparin (2 µg/mL)
96.8 mL	Neuronal cell culture medium (see table of materials)

Table 2. Components required for making 50 mL of NIM

Amount	Component
49 mL	DMEM/F-12
0.5 mL	N2 supplement (100X)
0.5 mL	MEM non-essential amino acids (100X)
2 µL	Heparin (2 µg/mL) (Stock Conc. is 50 mg/mL)

Table 3. Components required for making 50 mL of differentiation media

Amount	Component
1 mL	B-27 (50X)
500 µL	Antibiotic-Antimycotic (100X)
5 µL	Retinoic acid (0.1 µM)
50 µL	GDNF (10 µg/mL)
50 µL	BDNF (10 µg/mL)
5 µL	Ascorbic acid (0.2 µg/mL) (Stock Conc. is 2 mg/mL) NOTE: Recommended to be r
48.5 mL	NIM

nade fresh.

Table 4. Components required for making 200 mL of Antibody buffer

Amount	Component
1.75 g	NaCl (150 mM)
1.2 g	TrisBase (50 mM)
2 g	BSA 1%
3.6 g	L-lysine (100 mM)
8 g	Sodium Azide (4%)
200 mL	Distilled water. NOTE: Initially dissolve the required components in 150 mL of wa

ater, then adjust to 200 mL.

Table 5. Components required for making 3 mL of cell permeabilization and blocking solutio

Amount	Component
600 µL	20% Goat serum
6 µL	0.2% Triton-X100
2394 µL	Antibody buffer. NOTE: Initially dissolve the required components in 2 mL of Ab

n

buffer, and then adjust to pH 7.4. Then add more Ab buffer to adjust to final volume of 3 mL and filter st

terilize.

Table of Materials

Name of Material/Equipment	Company	Catalog Number	Comments/Descriptio
4-well Glass Chamber Slides	Sigma	PEZGS0816	
Alexa Fluor 488	Invitrogen	A-11001	
Alexa Fluor 594	Invitrogen	R37117	
Antibiotic-Antimycotic	Gibco	15240062	
Anti-β-Tubulin III	Thermo	MA1-118X	
B27	Thermo	17504001	
B27 - minus vitamin A	Thermo	12587010	
BDNF	PeproTech	450-02	
BSA	Sigma	A8531	
CellTiter-Glo	Promega	G7572	
CoolCell	Corning	432000	Cell freezing containers
CryoStor CS10	StemCell Technologies	7930	Cryopreservation medi
DAPI	Thermo	D1306	
DMEM/F12	Gibco	11320033	
DMSO	Sigma	34869-100ML	
EGF	Gibco	PHG0311	
FGF	Gibco	PHG6015	
Formaldehyde	Thermo	FB002	
GDNF	PeproTech	450-10	
Glutamax	Gibco	35050061	L-alanyl-L-glutamine su
Goat Serum	Thermo	50062Z	
Heparin	Calbiochem	375095	
Laminin	Sigma	L2020-1MG	
L-Ascorbic Acid	Sigma	A92902-25G	
L-lysine	Sigma	L5501	
MEM non-essential amino acids	Gibco	11140050	
mFreSR	StemCell Technologies	5854	Serum-free cryopreser
N2	Gibco	17502048	
NaCl	Sigma	71376	
Neurobasal Medium	Gibco	21103049	
Nunc 384-Well Polystyrene White Mic	Thermo	164610	
PBS	Thermo	10010-049	
Poly-L-lysine	Sigma	P5899-5MG	
ProLong Gold Antifade Mountant	Thermo	P10144	
Retinoic Acid	Sigma	R2625	
Sodium Azide	Sigma	S2002	
StemPro Accutase	Gibco	A1110501	Cell dissociation reager
Synaptophysin	Thermo	MA5-14532	
Tris Base	Sigma	10708976001	
Triton X-100	Sigma	X100-100ML	

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s ensuring standardized controlled-rate -1°C/minute cell freezing in a -80°C freezer
ium containing 10% DMSO

ıpplement

vation medium designed for the cryopreservation of human embryonic and induced pluripotent stem ce

ıt containing proteolytic and collagenolytic enzymes

ells

JoVE Reviwers' Response – 2nd cycle – Rebuttal letter

Dear Editor and Reviewers:

We would like to thank again the editor and referees, especially reviewer #2, for evaluating our manuscript. We have tried to address the reviewers' concerns in a proper way and believe that our paper has improved considerably.

Please find following our point-by-point response to the editorial and reviewers' comments.

Sincerely,

Amir Bagheri
March 26, 2020

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Editorial comments:

Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

**Some parts of the paper have been edited to address your concerns.
2. Please revise lines 43-45, 79-82, 165-167, 352-355 to avoid textual overlap with previously published work.

**Revisions have been made.
3. Keywords: Please provide at least 6 keywords or phrases.

**More keywords added.
4. All methods that involve the use of human or vertebrate subjects and/or tissue sampling must include an ethics statement. Please provide an ethics statement at the beginning of the protocol section indicating that the protocol follows the guidelines of your institution.

**Ethics statement has been added.
5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: falcon, Tryple Express, neurobasal, etc.

**Corrections have been made to address your concerns.
6. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.

**Corrections have been made to address your concerns.
7. 2.2. What happens after centrifugation? Do you aspirate the supernatant?

**Corrections have been made to address your concerns.
8. Section 6: Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE." Please ensure that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

**Corrections have been made wherever possible to address your concerns.
9. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

**Done
10. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

**Took into consideration while highlighting.
11. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

**Took into consideration while highlighting.
12. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

**The Disclosure statement has been added to address your concern.
13. Please remove the embedded figures and tables from the manuscript.

**Done
14. Figure 3 and Figure 4: Please use the micro symbol µ instead of u. Please include a space between all numbers and the corresponding unit: 0.1 µM, etc.

**Requested changes have been made.
15. Please include a scale bar, ideally at the lower right corner, for all microscopic images to provide context to the magnification used. Define the scale in the appropriate figure Legend.

**Except for the newly made Figure 6, other figures come *intact* from published papers and unfortunately, I do not have access to all the raw materials to make the requested changes.
16. Please upload each Table individually to your Editorial Manager account as an .xlsx file. Avoid any coloring or formatting in the tables.
**Done.

Reviewers' comments:
Reviewer #2:

Manuscript Summary:

The authors have substantially improved the manuscript. However, several of the original concerns have not been adequately adressed and the quality of language and grammar still needs improvement.

Listed below are some of the original concerns, how authors addressed them and remaining issues:

* "In the abstract the authors are mentioning that transformed cell lines are commonly used in compound screening assays and that they are aneuploidy and not able to reliably mimic human biology. Probably the authors refer to immortalized cell lines but exactly which cells the authors actually refer to is not clear and it is advisable to follow up this in the introduction with supporting references for clarity's sake. For instance, it can be misinterpreted that the authors refer to human pluripotent stem cell derived neurons that in general does not suffer from the mentioned limitations.

*** To address the reviewer's concern and for more clarification two valuable cell lines, iPSCs and ESCs, are discussed in the Discussion as alternative cell sources which could be used alongside hNPCs."

The authors are still writing about transformed cell lines in the abstract without following up which cells they refer to. Do they mean immortalized cell lines? As they agree, iPSC/ESC-derived cells are indeed valuable cell sources but do they mean that iPSC and ESC are the transformed cell lines? Please clarify which cells that possess shortcomings such as aneuploidy and inability to mimic human biology. It cannot be iPSC/ESC-derived cells as they are good alternatives to hNPC as the authors agree with.

Answer: To avoid any confusion, the sentence which the reviewer is referring to is changed in order to address more generally the need for more relevant cell model as follows:

“Nowadays, cell lines are widely used in compound screening assays in the field of neuroscience. While cell lines often differ genetically and phenotypically from their tissue origin, primary cells maintain the important markers and functions observed in vivo. Therefore, neurite outgrowth assay could considerably benefit from using human neural progenitor cells (hNPCs) as primary human cell model due to the great translational potential and physiological relevance that these cells could offer.”

* "Step 3.4: how is the dissociation process monitored? Bye eye? By microscopy? This needs to be clarified.

*** The dissociation will become visible to naked eye. Before dissociation, the neurospheres are in the form of spheres. But after submerging in dissociation reagent and by pipetting up and down they will become single."

For ease of use for users of the protocol add a note that the dissociation will become visible to naked eye.

Answer: Correction is made. Part 2.6: “NOTE: The dissociation will become visible to naked eye. Before dissociation, the neurospheres are in the form of spheres. But after submerging in dissociation reagent and by pipetting up and down they will become single.”

* "It should be mentioned that HNPCs represent young neural cells, even if they are further differentiated they still would not be representative of neurons from older individuals, considering age-related epigenetic changes

that occurs through life. Thus, for results from compound screening for neurodegenerative or age-related diseases, drugs or compounds that induce neuronal regeneration and repair might not have the physiological relevance that the authors more or less promise. The manuscript needs a discussion around this limitation. For research questions that concern neurodevelopment their model is of higher value.

*** To address the reviewer's concern, it is now stated in Discussion that the applications of the protocol presented in this paper covers a range of abnormalities from neurodevelopmental disorders to neurodegenerative diseases and psychiatric disorders."

In the current version of the manuscript the authors state that hNPCs constitute a desirable model system for nervous system function and development (line 355-356), which I do not object to. The point is that it is questionable how relevant hNPCs are for studying late onset diseases and degenerative disease. I cannot find anything written about that it's a good model for degenerative and psychiatric disorders in the discussion thus why are the authors claiming that they have done so here in the comment? To be clear, it should not be stated that hNPC is a good model system for late onset disease in the manuscript. Either the authors have misunderstood my comment or they do not agree with me.

Answer: To address the reviewer's concern the following sentence is added to Discussion:

"(IV) hNPCs could be considered as young neural cells, not representing age-related epigenetic modifications accumulating in cells throughout a person's life. As neurodegenerative disorders occur in older individuals, hNPCs may lack adequate physiological relevance to be an appropriate model for late onset conditions."

* "The discussion lacks comparison to why this method is advantageous compared to other neurite outgrowth assays. If I were going to start an experiment were the output measurement would be neurite length I don't know why I would use the presented method compared to others. In order to increase the possibilities that their method will be used a transparent comparison to other methods is required. The authors need to highlight the strengths with their method.

*** To our best knowledge this protocol would be considered among just few papers available to describe in detail the test for neurite length upon treatment with test compounds. Furthermore, describing in detail how to use hNPCs for neurite outgrowth assay would be unique."

The authors give themselves a disadvantage to not highlight the strengths of their method. I encouraged it previously and do it again by suggest to writing what they wrote as response to my comment: describing in detail how to use hNPCs for neurite outgrowth assay would be unique.

Answer: To address the reviewer's concern the following sentence is added to Discussion:
"This protocol is considered among a few available papers describing the test for neurite length upon treatment with test compounds. Furthermore, describing in detail how to use hNPCs for neurite outgrowth assay and neurotoxicity assessment would be unique."

* "The authors discuss around that high precision pipetting skills is required if neurospheres would be plated as single cells for high-throughput toxicity assays (line 262-267, now 365-371). This is not necessarily true since FACS could solve this issue.

*** We have tried to keep the protocol simple to follow and possible to perform using the equipment usually

available in labs."

It is good to keep the protocol simple but by not mentioning FACS that most researchers would think could solve this issue, reduce the trust of the authors. If it is difficult to FACS hNPC, low survival etc then it would be good for the readers to know. But if there is no problem to FACS hNPC you have a solution to a drawback to one of the applications for the protocol.

Answer: To address the reviewer's concern the following sentence is added to Discussion:

"Moreover, bulk cell sorting by FACS (Fluorescence Activated Cell Sorting) could also potentially be used to accurately separate the required number of cells circumventing the need for high precision pipetting skills."

Reviewer #4:

Manuscript Summary:
This study describes that neurite outgrowth assay and neurotoxicity analysis were investigated with authoress's developed protocol, which is provides analysis of neuronal morphology with quantitative measurements of modifications on neurite length and synaptic protein localization in a manner of cell by cell. Authors applied the protocol to evaluate effects of small-molecule compounds on neurite outgrowth and neuronal cell counts. In addition, the analysis showed HDAC inhibitors significantly extended neurite length without neurotoxicity. These findings suggests that the newly develop protocol could analyze quantitative measurements of neurite outgrowth in human cells.

Major Concerns:
The point is not clear. It is not clear how the author's method has changed. In abstract, neither the background, purpose, summary of the results nor conclusions are given as I wrote above.

Answer: Due to editorial and reviewers' comments, I believe that the contents of the paper have greatly improved. However, considering the improvements, if the Reviewer #4 is not still satisfied with corrections I would appreciate if he/she could more clearly explain his/her requests as I am having difficulties understanding the points he/she is referring to in Major Concerns part of his/her comments. Based on my best understanding, I revised the Abstract to better address the reviewer's request.