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Post-differentiation replating of human pluripotent stem cell-derived neurons for high-content screening of neuritogenesis and synapse maturation

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May 7th, 2019

Dear Editor,

We have submitted revisions to our paper entitled "Post-Differentiation Replating of Human Pluripotent Stem Cell-Derived Neurons for High-Content Screening of Neuritogenesis and Synapse Maturation". We have addressed all editor's querys.

Reviewer 1 was satisfied with our previous responses, and had no further comments, other than to ask if we might provide additional advice on how to improve the uniformity of cell distribution. We have now included a statement pertaining to this (lines 270-274).

We have addressed where possible the additional comments of Reviewer 2. We have added statistical analyses to the viability graphs in Figures 1 and 2. We have emphasized that the extended incubation with proteolytic enzyme produces a doubling of cell viability after neuronal cultures are replated – both immediately and for many days thereafter. The previous manuscript revisions added multiple experimental approaches to thoroughly document this viability improvement. In our estimation, the iPSC research community would find that such a doubling of cell recovery and viability would be quite valuable, in addition to the general value of a video demonstration for iPSC-derived neuronal culture replating.

At this time we respectfully request that you make a decision at the Editorial level regarding the suitability of our paper for JoVE. We do not wish to engage further with Reviewer 2, as this this reviewer continues to demonstrate a fundamental misunderstanding of the techniques we describe and the basic biological premises underlying them. We would greatly appreciate your decision within two weeks. In case you believe that this work remains unsuitable for JoVE, this would allow us to quickly resubmit to another methods journal in the field.

Sincerely,

Shelley Halpain, Ph.D.
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TITLE:

Post-Differentiation Replating of Human Pluripotent Stem Cell-Derived Neurons for High-Content Screening of Neuritogenesis and Synapse Maturation

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KEYWORDS:

iPSC, stem cells, replating, neuritogenesis HCS, viability, neuron, neurite outgrowth, synapses, microscopy

SUMMARY:

This protocol describes a detailed procedure for resuspending and culturing human stem cell derived neurons that were previously differentiated from neural progenitors in vitro for multiple weeks. The procedure facilitates imaging-based assays of neurites, synapses, and late-expressing neuronal markers in a format compatible with light microscopy and high-content screening.

ABSTRACT:

Neurons differentiated in two-dimensional culture from human pluripotent stem-cell-derived neural progenitor cells (NPCs) represent a powerful model system to explore disease mechanisms and carry out high content screening (HCS) to interrogate compound libraries or identify gene mutation phenotypes. However, with human cells the transition from NPC to functional, mature neuron requires several weeks. Synapses typically start to form after 3 weeks of differentiation in monolayer culture, and several neuron-specific proteins, for example the later expressing pan-neuronal marker NeuN, or the layer 5/6 cerebral cortical neuron marker CTIP2, begin to express around 4–5 weeks post-differentiation. This lengthy differentiation time can be incompatible with optimal culture conditions used for small volume, multi-well HCS platforms. Among the many challenges are the need for well-adhered, uniformly distributed cells with minimal cell clustering, and culture procedures that foster long-term viability and functional synapse maturation. One approach is to differentiate neurons in a large volume format, then replate them at a later time point in HCS-compatible multi-wells. Some main challenges when using this replating approach concern reproducibility and cell viability, due to the stressful disruption of the dendritic and axonal network. Here we demonstrate a detailed and reliable procedure for

enzymatically resuspending human induced pluripotent stem cell (hiPSC)-derived neurons after their differentiation for 4−8 weeks in a large-volume format, transferring them to 384-well microtiter plates, and culturing them for a further 1−3 weeks with excellent cell survival. This replating of human neurons not only allows the study of synapse assembly and maturation within two weeks from replating, but also enables studies of neurite regeneration and growth cone characteristics. We provide examples of scalable assays for neuritogenesis and synaptogenesis using a 384-well platform.

INTRODUCTION:

Human pluripotent stem cell (hiPSC)-derived neurons are increasingly relevant in the areas of basic research, drug development, and regenerative medicine. Workflows and procedures to optimize their culture and maintenance, and improve the efficiency of differentiation into specific neuronal subtypes, are evolving rapidly[1,2]. To improve the utility and cost-effectiveness of human stem cell-derived neurons as model systems amenable to high-content analyses in drug discovery and target validation, it is useful to decrease the culturing time required to generate mature, functional neurons, while retaining maximum robustness, reproducibility, and phenotype relevance. Although 3-dimensional organoid cultures are driving breakthroughs in neurodevelopment research[3], 2-dimensional monolayer cultures are especially compatible with automated imaging-based applications due to their minimal tissue thickness.

However, the adaptation of imaging-based screening methods to models of human neurological and neurodevelopmental disease faces a major challenge. The protracted timeframe over which the human nervous system matures in vivo necessitates extended time in culture to accommodate natural programs of gene expression and achieve neuronal maturation.

One practical consequence of the lengthy neuronal differentiation program is that the maintenance of hiPSC-derived monolayer cultures must be sustained for many weeks to achieve adequate synapse maturity. During this time, neural progenitors that remain undifferentiated continue to divide. These can quickly overgrow the culture and usurp the nutrient content required to maintain viable postmitotic neurons. Vigorously dividing neural progenitor cells (NPCs) can also compete with neurons for the growth substrate. This can render such cultures subject to problems of poor adhesion, a condition unsuitable for imaging-based assays. Moreover, many investigators find that the smaller the culture volume, the greater the difficulty in maintaining healthy populations of differentiated neurons long enough to observe the late stages of neuronal differentiation. In other words, assays of synapse maturation using high content screening (HCS) approaches can be very challenging for human-derived neurons.

To circumvent some of these problems, a procedure of resuspending and replating previously differentiated hiPSC-derived neurons has been used. Firstly, it allows the study of neurite outgrowth (or, more accurately, neurite regeneration) in a population of fully committed neurons. Secondly, the replating of previously differentiated neurons from a large volume format (like 10 cm plates or larger), down to small volume formats (like HCS-compatible 96- or 384-well microtiter plates) enables a significant reduction in total culturing time in the small volume condition. This facilitates the study of synapse assembly and maturation over subsequent weeks

in vitro.

However, the replating of mature neurons that have already established long neurites and a complex connectivity network presents several challenges, one of which is the sometimes high and variable rate of cell death. Here, we describe a replating procedure that results in excellent cell survival and reproducibility. Commonly, neurons are exposed to proteolytic enzymes for short incubation periods (typically ~3−10 min) in order to detach cells from the substrate prior to trituration. This brief proteolysis time is customarily used for resuspending and passaging many types of dividing cells, including non-neuronal cells and undifferentiated progenitors[4-6]. However, for differentiated neurons bearing long, interconnected neurites, it is essential not only to detach cells from the substrate but also to disrupt the dendritic and axonal network in order to isolate individual cells while minimizing damage. Indeed, a thick meshwork of neurons usually tends to detach from the substrate as a single sheet, rather than as individual cells. If care is not taken to loosen the thick network of neurites, neurons not only become irreversibly damaged during trituration, but many of them fail to pass through the filter used to remove clumps, resulting in poor cell yield. Below we describe a simple modification to a widely-used protease incubation procedure to counter these difficulties.

In the protocol described below, neurons are incubated for 40−45 min with a mild protease, such as the proteolytic enzyme (e.g., Accutase). During the first 5−10 min after adding the enzyme, the neuronal network lifts off from the substrate as a sheet. Incubation with the protease proceeds for an additional 30−40 min before proceeding with gentle trituration and filtering. This extra incubation time helps ensure that the digestion of the material relaxes the intercellular network, thereby ensuring that subsequent trituration produces a suspension of individual cells. This procedure maximizes the uniformity of cell distribution upon replating while minimizing cell death. We have successfully applied this replating method to hiPSC-derived neuronal cultures generated by various differentiation protocols[7,8] and from various lines of hiPSCs. The procedure is nominally suitable for use with most or all lines of stem cell-derived neurons. We have observed that an extended protease incubation time is not absolutely essential for replating cultures from small format plates (e.g., 35 mm diameter); however, as we show here, it provides a significant benefit when replating from large diameter plates (e.g., 10 cm diameter or larger), probably because neurites in such plates can extend very long processes and form a densely interconnected array.

Here we demonstrate this method and briefly illustrate its application in assays for early neuritogenesis and for synapse maturation, which involves clustering of pre- and postsynaptic proteins along the dendrites and axons, followed by their later colocalization at synaptic sites. The examples highlight the advantages this protocol offers in preserving cell viability and reproducibility. First, it permits investigators to study early steps in human neuritogenesis. The experimental setting is similar to the commonly used primary cultures of rodent cortical or hippocampal neurons, where cells are extracted from late fetal or early postnatal brain, dissociated by trituration after gentle protease treatment, and allowed to initiate neurites or to regenerate neurites that were severed in the procedure[9,10]. Similar to such rodent primary neurons, hiPSC-derived neurons begin to form or regenerate their neurites within hours after

replating, allowing imaging of growth cones and neurite morphology in an environment optimal for high spatiotemporal imaging with fewer surrounding undifferentiated cells. We have observed that neurite outgrowth is more synchronized compared to the variable delays and different outgrowth rates seen when neurons first begin to differentiate from a progenitor population. In addition, replating enables assays of neurons expressing neuronal subtype markers that typically appear later in neural development, such as the cortical layer 5/6 transcription factor CTIP2 (Chicken ovalbumin upstream promoter transcription factor-interacting protein 2), or the pan-neuronal marker NeuN[11]. An especially useful feature of the replating approach is that synaptogenesis proceeds within a time frame compatible with HCS.

PROTOCOL:

1. Differentiation period prior to replating

1.1. Differentiate neurons on 10 cm dishes, using a protocol of choice[7,8] until neurons have formed a thick network with their processes and express not only early neuronal markers such as MAP2 or TuJ1, but also late markers such as NeuN.

1.2. Change half the medium of choice every 4 days during the neuronal differentiation process.

NOTE: More extensive or frequent medium changes dilute essential trophic factors, and could disfavor maturation.

1.2.1. For the iPSC-derived WT126 neurons, use the following post-differentiation culturing media: 5 mL 100x N2 supplement, 10 ng/mL BDNF, 10 ng/mL GDNF, 1 µg/mL laminin, 200 µM ascorbic acid, 1 µM dibutyryl-cAMP and 10 mL SM1 for 500 mL Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F12). Gradually, using half-media changes, replace with neural basal medium (**Table of Materials**) and the same supplements.

1.2.2. For the iPSC-derived CVB WT neurons, use the following post-differentiation culturing medium: 500 mL neural basal A medium (**Table of Materials**) and 500 mL DMEM/F12 medium with 2 µg/mL laminin, 10 mL glutamine supplement (**Table of Materials**), 0.75 mg/mL sodium bicarbonate, 5 mL minimum essential medium (MEM) nonessential amino acids, 0.2 mM ascorbic acid, 10 ng/mL BDNF, 20 mL 50x B27, and 10 mL 100x N2 supplement.

2. Coating multiwells

2.1. The day before replating, coat with poly-L-ornithine (PLO). Dissolve PLO in sterile water to make a stock solution (10 mg/mL). Store this stock at -20 °C. Dilute PLO 1:100 in water to yield a concentration of 50 µg/mL when coating glass and 1:1000 ratio (10 µg/mL) when coating plastic.

2.2. Apply the coating directly to the target plates. Use the volume of coating appropriate to the plate size (i.e., for a 24-well plate apply 500 µL of PLO solution per well).

2.3. Allow plates to sit in the dark overnight at room temperature.

2.4. Retrieve coated plates on the day of replating and transfer to a sterile biosafety cabinet.

2.5. Aspirate the PLO solution and rinse twice with sterile water.

2.6. Dilute laminin (1.15 mg/mL) in phosphate-buffered saline (PBS) at 1:400 dilution.

NOTE: Thaw laminin at 4 °C and quickly add to PBS to avoid aggregation of laminin and uneven coating.

2.7. Aspirate sterile water and apply 500 µL of laminin coating to wells previously coated with PLO.

2.8. Place plates in a 37 °C incubator for a minimum of 4−6 h. Use longer incubations, up to 16 h, for glass surfaces. Use consistent incubation times.

3. Replating differentiated neurons

3.1. Rinse plate of differentiated neurons with PBS once gently. Disperse PBS gently down the wall of the plate, and not directly onto the cells, to avoid disrupting them.

3.2. Gently aspirate PBS, being careful to avoid touching the cells directly but to aspirate from the edge of the dish while tipping it towards the researcher.

3.3. Apply at minimum 1 mL of the proteolytic enzyme (**Table of Materials**) per 10 cm plate and return cells to incubator. Add slightly higher volumes if the tissue culture room exhibits a high evaporation rate due to low humidity.

3.4. Incubate cells with proteolytic enzyme for 40−45 min in order to detach them from the plate and to detach them from other neurons within the neuronal network.

NOTE: Timing at this step is critical. Quenching the protease too early can lead to increased cell death after replating. The proteolytic enzyme manufacturer recommends that temperatures much lower than 37 °C be used with longer incubation periods for passaging cell lines (e.g., overnight at 4 °C). However, handling neurons at 4 °C should be avoided, as they often show poor survival after cold exposure. The manufacturer also states that a 60 min incubation with the enzyme at 37 °C leads to its enzymatic inactivation. However, in authors' experience, a 40−45 min incubation of hiPSC-derived neuronal cultures at 37 °C is sufficient for efficient dissociation and excellent neuronal survival upon replating.

3.5. Check neurons on a phase-contrast microscope during the incubation time and allow protease treatment to continue until the neural network completely detaches from the plate and starts to break apart in smaller sheets upon briefly shaking the plate under the microscope.

3.6. Quench the protease activity using 5 mL of fresh DMEM media per 1 mL of protease in the 10 cm plate to stop the digestion. Gently triturate cells against plate 5–8 times to disrupt network, using serological pipettes. Be careful not to apply too much pressure when triturating, as differentiated neurons are fragile. Do not use a P1000 tip, as the end is too sharp and narrow, and thus can sheer or damage the neurons.

3.7. Apply solution with cells through a cell strainer with 100 µm diameter mesh into a fresh 50 mL conical tube drop by drop.

3.8. Rinse strainer with an additional 5 mL of fresh DMEM media, after cells have filtered through.

3.9. Spin cells in benchtop centrifuge at 1,000 x *g* for 5 min.

3.10. Return conical tube to the biosafety cabinet and aspirate most of the media, leaving around 250 µL to ensure cells retain moisture.

3.11. Resuspend cells gently in 2 mL of fresh DMEM media. Do not pipette the pellet against the side of the tube. Instead, gently invert conical 2–3 times and pass cells through the end of a 5 mL serological pipet to dislodge them.

3.12. Apply 10 µL of resuspended neurons onto the edge of a hemocytometer.

3.13. Add 8–10 µL of trypan blue to droplet of cells to assess cell viability during this resuspension step. Apply 10 µL of this mixture to the hemocytometer or other automatic cell counters. Assess as viable those cells that are phase bright and exclude the trypan blue dye.

3.14. Determine amount of viable cells/mL, and prepare to dilute the contents of the conical tube according to the desired cell density.

3.15. Plate ~10,000 cells per well for a 384-well plate; plate ~150,000–200,000 cells per well for a 24-well plate.

3.16. Add fresh DMEM to the conical tube of resuspended cells to achieve appropriate dilution and add additional appropriate supplements such as B27 and/or BDNF, depending on the requirements of the specific cell line.

3.17. Gently tilt conical tube to mix 2–3 times.

3.18. Aspirate laminin coating from the 24-well plate, or from the 384-well multiwell plate using a 16-channel pipet and rinse once with PBS.

3.19. Aspirate PBS using a P1000 for the 24-well plate, or the 16-channel pipette for 384-well plates.

3.20. Apply cell solution to each well in a figure eight motion to avoid clumping. Plating uniformity might also be optimized by using automated liquid handling devices.

NOTE: The addition of laminin to the media starting 2−4 days post-replating also helps maintain a homogenous distribution of the cells.

3.21. Repeat steps 3.19 and 3.20 for each well.

3.22. Return the plate to incubator set at 37 °C and 5% CO_2.

3.23. After 2 days, start changing half the medium every 4 days using the post-differentiation culturing media described in section 1.2. After the desired maturation time, fix cultures using 3.7% formaldehyde at 37 °C and stain cells according to the experimental requirements.

4. Cell viability assays post-replating

4.1. Add the early cell death reporter (VivaFix: 0.5 µL of the 50 µL stock solution per 500 µL culture media per well) after briefly vortexing the stock solution.

4.2. Image the live cells cultured on glass-bottom multiwells, after 20 min, without any washing step, since the dye fluoresces only once inside the cells, or fix and co-stain with 4',6-diamidino-2-phenylindole (DAPI) and/or other antibodies before imaging using a confocal microscope.

5. Immunostaining

5.1. Fix cultures with 3.7% formaldehyde in PBS plus 120 mM sucrose for 20 min at 37 °C.

5.2. Rinse and permeabilize with 0.2% Triton X-100 for 5 min at room temperature, and then block for 30 min with 2% bovine serum albumin (BSA).

5.3. Aspirate the BSA without rinsing and incubate for 1 h at room temperature with rabbit anti-MAP2 antibody 1:1000, mouse anti-β3 tubulin (TuJ1) antibody 1:2000, chicken anti-NeuN antibody (1:100) and rat anti-CTIP2 antibody (1:500), or with mouse anti-PSD95 (1:200), rabbit anti-synapsin 1 (1:200) and chicken anti-MAP2 antibody for synaptic staining.

5.4. Rinse with PBS, and incubate with Alexa Fluor-conjugated secondary antibodies plus DAPI for 45 min at 37 °C.

5.5. Finally, wash twice with PBS before imaging.

6. Calcium imaging

6.1. Infect cultured cells with AAV8-syn-jGCAMP7f-WPRE (The Salk Institute for Biological

Studies, GT3 Core Facility) at 10 days post-replating and image to assess spontaneous calcium transients at 3−4 weeks post-replating.

7. Image acquisition and Analysis

NOTE: For details on the acquisition system please refer to Calabrese et al.[12].

7.1. Use an imaging module for manual or automated image acquisition, and an image analysis software module, such as CellProfiler[13], for morphometric measurements.

7.2. Calculate average length of TuJ1 positive neurites and MAP2 positive dendrites by measuring total length per field of view divided by the number of neurons (DAPI + MAP2 or TuJ1 positives cells) within the same field.

REPRESENTATIVE RESULTS:

The replating of hiPSCs-derived neurons that have been differentiated for multiple weeks offers several advantages. However, detaching and replating differentiated neurons that have long, interconnected dendrites and axons (**Figure 1A**) can result in a high fraction of irreversibly damaged neurons.

As described in the protocol section, we used incubation with a proteolytic enzyme to detach the neurons from the substrate. Typically, due to their thick meshwork of neurites (**Figure 1A**), the cells tend to detach all at once as a single syncytial-like layer, which often begins to float in the well (**Figure 1C**). This happens fairly quickly, and is perhaps why most laboratories tend to collect the cells after only 5 min of incubation with their proteolytic enzyme of choice, and to immediately break apart the sheet of cells by trituration (pipetting it up and down). However, this mechanical manipulation appears to result in high stress for neurons. We believe the degree of stress may be proportional to the area covered by the neuronal network, because we find that the apparent damage from inadequate protease incubation is greater for 10 cm or larger plates than for 35 mm or smaller plates. Thus, counterintuitively, we found that prolonging the enzymatic incubation to 40−45 min reduces cell death at the time of cell dissociation (**Figure 1C**) and permits more efficient recovery of live, healthy cells that emit processes over hours to days post-replating (**Figure 1B**). Presumably, the extended incubation time with a mild proteolytic enzyme allows the partial digestion of proteins that strongly adhere cells and their processes to one another and to the extracellular matrix. This allows the resuspension process to work efficiently to separate individual cells without the need for overly vigorous trituration.

To quantify the effectiveness of the extended enzyme incubation procedure, we evaluated viability of suspended cells immediately after trituration using trypan blue (**Figure 1C**), and later at 1, 3 and 7 days post-replating using an early cell death marker (**Figure 2**). Immediately after trituration, trypan blue staining indicated that there was substantially greater cell death after a 5 min incubation compared to a 45 min incubation with the proteolytic enzyme (**Figure 1C**). The two iPSC lines we used to illustrate this observation were differentiated into neurons from the neural progenitor stage using different protocols, and showed broadly different sensitivity to the

replating procedure. Cultures from the WT126 line differentiated into NPCs using the "rosette selection method"[7] were more sensitive to damage than cultures differentiated from the CVB WT24 line using a rapid differentiation method[8]. Nevertheless, for both lines the degree of immediate cell death was approximately halved by using the extended enzyme incubation procedure.

After replating, cultures exhibited an approximate doubling of cell viability over subsequent days using the extended enzyme incubation procedure, as determined by the density of DAPI-positive cells (**Figure 2B**, graph on the left). In addition, the extended enzyme incubation resulted in a lower density of dead or dying cells detected using a dye-exclusion viability kit (**Figure 2B**, graph on the right). The fraction of dead cells detected using the viability assay after 24 hours post-replating was higher than that seen using trypan blue immediately following trituration. This probably reflects the accumulation of dead and dying cells over these first 24 hours, although it is also possible that the viability assay more sensitively detects cell death than the trypan blue assay. Dead cells continued to be detected over 1−7 days post-replating (**Figure 2B**). The initial plating density for both experimental groups (5 min and 45 min) was identical and based on hemocytometer live cell counts of the post-trituration cell suspension. Importantly, at all time points post-replating, the number of live, healthy cells was approximately doubled following the 45 min enzyme incubation compared to the 5 min incubation. These observations were confirmed qualitatively using phase contrast microscopy to monitor cell morphology at every step: before replating, during replating, and after replating (**Figure 1** and **Figure 2**).

One of the advantages of replating hiPSC-derived neurons after they have been differentiating for several weeks is that most of the cells will have exited the progeitor stage and become committed to a neuronal phenotype at the time of replating. The transition phase of neuronal differentiation from neural progenitors takes place over several days, with greater numbers of cells gradually expressing early-stage neuronal markers, such as beta-III tubulin (detected by antibody TuJ1) or MAP2. Gene expression evolves over several weeks, eventually resulting in expression of late-stage pan-neuronal markers, such as NeuN, or cell-type specific markers for cortical neurons such as CTIP2. Therefore, the replating of previously differentiated hiPSC-derived neurons allows one to study early and transient neuronal events, such as neurite initiation, in identified subtypes of neurons. **Figure 3** illustrates the immediate presence of early neuronal markers in cultures that were replated after 4 weeks of pre-differentiation in a larger culture dish. Note that NeuN- and CTIP2-expressing neurons are readily identified within a few days post-replating (**Figure 3**). Characteristics and timing of neuronal differentiation can vary among hiPSC lines. For the WT 126 line and the CVB WT24 lines we describe here, 85 ± 12% (n = 2) and 52 ± 11% (n = 5) of the cells, respectively, expressed immunoreactivity for the βIII-tubulin marker TuJ1 at 4 days after replating using the extended protease incubation procedure. For both lines, 30−40% of the cells also express the layer 5/6 neocortical marker CTIP2. In addition to improved viability, the extended protease protocol also moderately enhanced neurite outgrowth (average neurite length per neuron), as shown in **Figure 4**. Both total neurite length (quantified using antibody Tuj1, which stains both axons and dendrites; **Figure 4B**, left graph) and dendrite growth (quantified using MAP2, which stains only dendrites; **Figure 4B**, center graph) were favored when using the extended protease incubation procedure. Note that the graph for DAPI-

positive (DAPI (+)) cell counts in **Figure 2** are based on the same image samples as the cell count graph in **Figure 4**, but in **Figure 2** they were quantified using a non-automated method assisted by Fiji software, while in **Figure 4** they were quantified using the automated image analysis software platform CellProfiler. These two image analysis approaches yielded similar results.

Replating is useful not only to study neurite outgrowth, but also to study synapses or other later-appearing biological features of neurons. Indeed, after just one week post-replating we observe markers for many presynaptic and postsynaptic proteins decorating the neuronal dendrites in a punctate pattern (**Figure 5A**). After 4 weeks we also begin to detect their colocalization, which is an indicator of the formation of functional synapses (**Figure 5A**). Moreover, electrical activity from spontaneous depolarization and synaptically driven currents is detectable using calcium imaging (**Figure 5B**) or multielectrode arrays (MEAs).

FIGURE LEGENDS:

Figure 1: Superior preservation of neuronal viability after longer incubation with the proteolytic enzyme for cell dissociation. (A) Phase contrast image of NPC-derived neurons differentiated for 3 weeks. The boxed region in the left image is enlarged at right. At this stage neurons have long processes, which form a thick meshwork. Scale bars: 80 µm (left); 35 µm (right). **(B)** Selected images of hiPSC-derived neurons at 1 and 5 days post-replating. Scale bar: 100 µm. **(C)** Left: cells detach as a single sheet within a few minutes after initiating protease treatment. Scale bar: 200 µm. Right: after trituration cells are counted on the hemocytometer before replating. Graphs show quantification of non-viable, trypan-blue positive cells after 5 min or 45 min incubation with the proteolytic enzyme for two different lines CVB WT24 (*** $p < 0.0003$, unpaired t-test) and WT 126 (*** $p < 0.0001$, unpaired t-test). Values represent the mean ± S.E.M of 4 individual replicates.

Figure 2: Neuronal viability after several days post-replating. (**A**) Images of WT126 NPC-derived neurons at 1 and 3 days post-replating using 5 min and 45 min protease incubation. Dying cells are labeled with the sensitive early cell death reporter. Corresponding brightfield images are shown on the left. Some cellular debris (blue arrow) is typically detected after replating, especially in the 5 min group. Scale bars: 150 µm. (**B**) Images of CVB WT24 NPC-derived cultures at 1, 3 and 7 days post-replating using 5 min and 45 min protease incubation. Dying cells are labeled with the sensitive early cell death reporter (red). All nuclei of living and dying cells are labeled with DAPI (cyan). The merged white signal represents DAPI and VivaFix-positive (+) cells. A few cells display VivaFix staining but lack DAPI staining (red cells in the combined image on the right); these are likely cells that were dead for many hours. Scale bars: 25 µm. Right graph shows quantification of the fraction of dead cells (VivaFix/DAPI) after different incubation time with the proteolytic enzyme (5 min vs 45 min: * $p < 0.05$ 1 d and *** $p < 0.001$, 3 d; two-way ANOVA, followed by multicomparison Bonferroni post hoc test). Left graph shows changes in overall cell density (# DAPI(+) cells for 5 min vs 45 min: *** $p < 0.0001$ for all time points; two-way ANOVA, followed by multicomparison Bonferroni post hoc test). All values are shown as the mean ± S.E.M of 3 replicates, with a minimum of 1500 cells scored per condition.

Figure 3: Detectable expression of late-stage neuronal markers immediately after replating. Cultures of CVB WT24 hiPSC-derived cells imaged 4 days after replating, using 45 min protease incubation, from a 10 cm dish that had been differentiated from the NPC stage for 4 weeks. Examples shown were stained for the pan-neuronal markers TuJ1, MAP2, or NeuN; or the deep-layer cortical pyramidal neuron marker CTIP2. Note the presence of extensive neurites, and the robust expression of TuJ1 and MAP2. Note especially that a substantial fraction of the neurons also expresses late-stage markers NeuN and CTIP2. Scale bar: 16 µm.

Figure 4: Neurite and dendrite growth over time following shorter and longer enzyme incubation during replating. (**A**) Replated CVB WT24 hiPSC-derived neurons quickly extend neurites, as detected using antibody Tuj1. Note that the extended protease incubation time promotes neuritogenesis. Scale bar: 25 µm. (**B**) Quantification of neurite and dendrite length, over 1, 3 and 7 days post-replating using either 5 min or 45 min protease. Total neurite length was quantified from TuJ1 (βIII-tubulin) staining; dendrite-specific staining was quantified from MAP2 staining snf corrected for the overall cell density (right graph). All values are shown as the mean ± S.E.M of 3 replicates. Neurite length 5 min vs 45 min: * $p < 0.05$ at 1 d and 7 d, ** $p < 0.01$ at 3 d; dendrite length 5 min vs 45 min: ** $p < 0.01$ at 1 d and 3 d, not significant (ns) at 7 d; # DAPI(+) 5 min vs 45 min: *** $p < 0.0001$ for all time points; two-way ANOVA, followed by multicomparison Bonferroni post hoc test.

Figure 5: Synaptogenesis and spontaneous calcium transients in differentiated hiPSC-derived neurons after replating. (**A**) Left: at 4 weeks post-replating using the extended protease incubation protocol, the presynaptic marker synapsin 1 is detectable in punctate clusters along the MAP2 positive dendrites. Scale bars: 5 µm. Right: selected dendritic region showing colocalization between presynaptic and postsynaptic clusters (yellow arrow), an indication of potentially active synapses. Scale bars: 1.5 µm. (**B**) Left: hiPSC-derived neurons infected with AAV8-syn-jGCaMP7f-WPRE were used to monitor spontaneous calcium transients driven by network activity. The brightfield image is shown adjacent to the pseudocolor rendering of GCaMP7 fluorescence at a single time point in the time-lapse series. Scale bar: 25 µm. Colored numbers indicate the 4 selected cells in which GCaMP7 fluorescence was measured over time, as shown in the traces on the right. Each colored trace corresponds to one cell. The asterisk points to the time during the live recording to which the image on the left corresponds.

Figure 6: Workflow of replating procedures to culture hiPSCs for high content screening and/or detailed studies of differentiation and neurite outgrowth or MEA recordings. Starting from a differentiated culture of neurons grown for 4 or more weeks in a larger format (e.g., a 10 cm culture dish), neurons are incubated with a protease for 40−45 min, triturated gently to dissociate and resuspend. Cells are then distributed into multi-wells compatible with HCS, replated onto substrates compatible with imaging growth cones (yellow arrow) or other structures, or onto multi-wells suitable for multielectrode array recordings. Scale bars from left to right: 27 µm, 5 µm, 2.5 µm, 130 µm.

DISCUSSION:

We have demonstrated a straight-forward procedure for the resuspension and replating of human neuronal cultures that optimizes viability, differentiation success, and subcellular imaging in a manner that is compatible with high content screening platforms, and other assays relevant to drug discovery. **Figure 6** illustrates the overall workflow and examples of such applications.

Although here we focused on hiPSC-derived neurons that are directed toward a cortical neuron fate, we expect that this method should be equally applicable to human embryonic stem cell-derived neurons, and to stem-cell derived neurons directed toward other neuronal phenotypes[14-18]. Moreover, we postulate that this extended protease procedure might be beneficial to other situations that require resuspension of cells having an established neurite meshwork, for example for FACS sorting or single-cell analyses of primary neurons[19,20].

Replating hiPSC-derived neurons provides a viable model system in which to investigate cellular and molecular mechanisms of many key biological events. For example, this procedure can facilitate detailed studies of human neurite outgrowth and growth cone characteristics, and comparison of findings to the extensive knowledge base built from decades of studying other species, both vertebrate and invertebrate. We find that the replating procedure has made it is easier to optimize conditions to generate larger growth cones that are well-suited for optical evaluation of subcellular structure and function (**Figure 6**). By comparison, the growth cones more typically observed during the initial differentiation of neurons from hiPSCs tend to be small and compact, and the dense array of non-neuronal cells in such cultures makes it difficult both to adjust substrate conditions to promote growth cone spreading and to image individual growth cones within the complex tissue environment. Thus, replating neurons onto a fresh dish provides a "cleaner slate" on which to view growth cones under good imaging conditions.

Replating is particularly advantageous when using cell culture platforms suitable for HCS because it greatly reduces the total time in which cultures are grown in small-volumes. The small working volumes of the individual wells of 96, 384 or 1536-well multi-wells (approximately 100, 50 and 5 microliters working volume, respectively) usually requires frequent (i.e., daily) media changes to counteract evaporation and nutrient depletion. Frequent media changes are costly, not only in terms of reagents and labor, but they can compromise the viability of cultures by diluting conditioned media and by increasing the chances that cells detach from the substrate due to mechanical turbulence. Replating of hiPSCs can also facilitate the recording of physiological activity using multielectrode arrays[21,22], or live imaging of cultures in assays of dynamic neuronal phenotypes[23].

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DISCLOSURES:

The authors have nothing to disclose.

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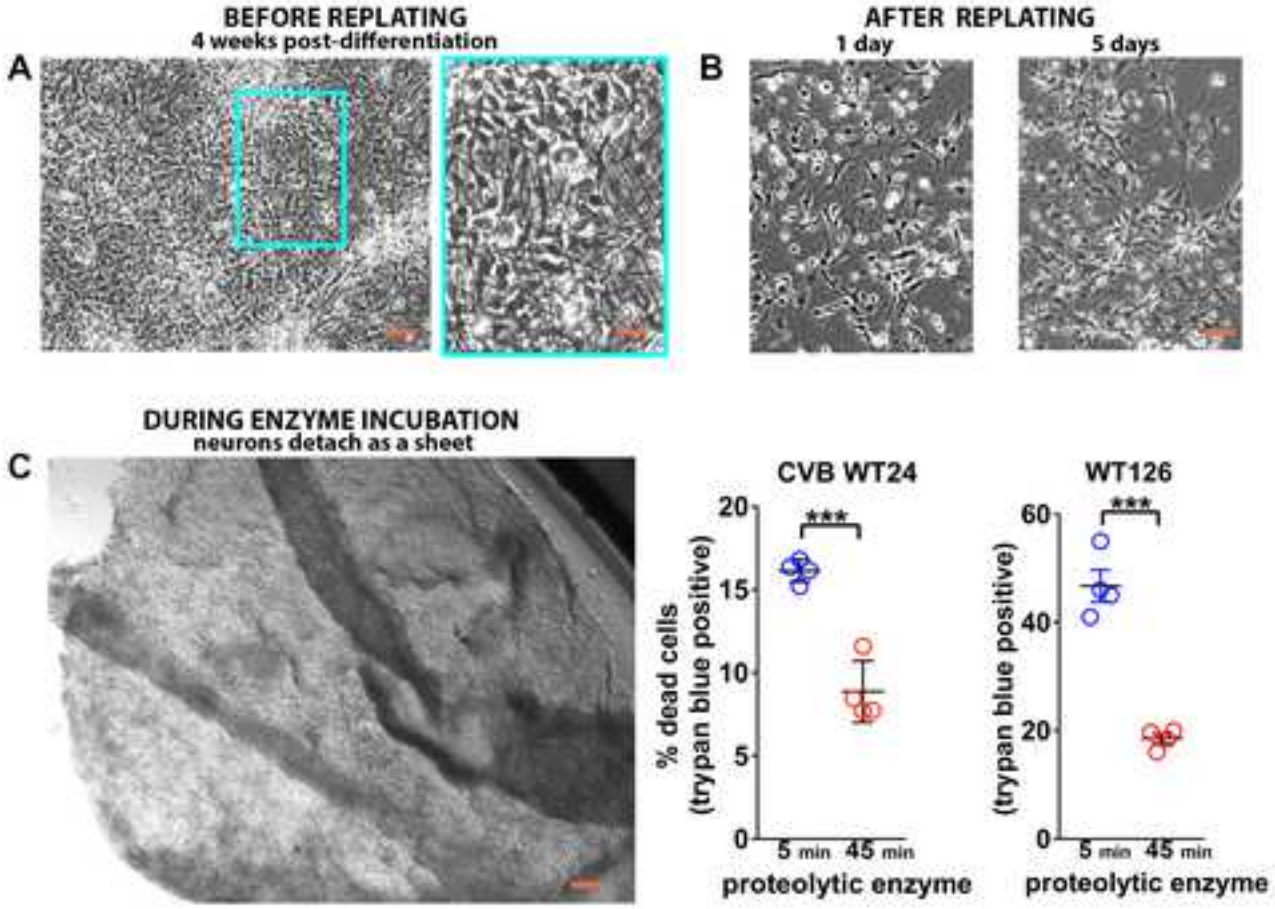

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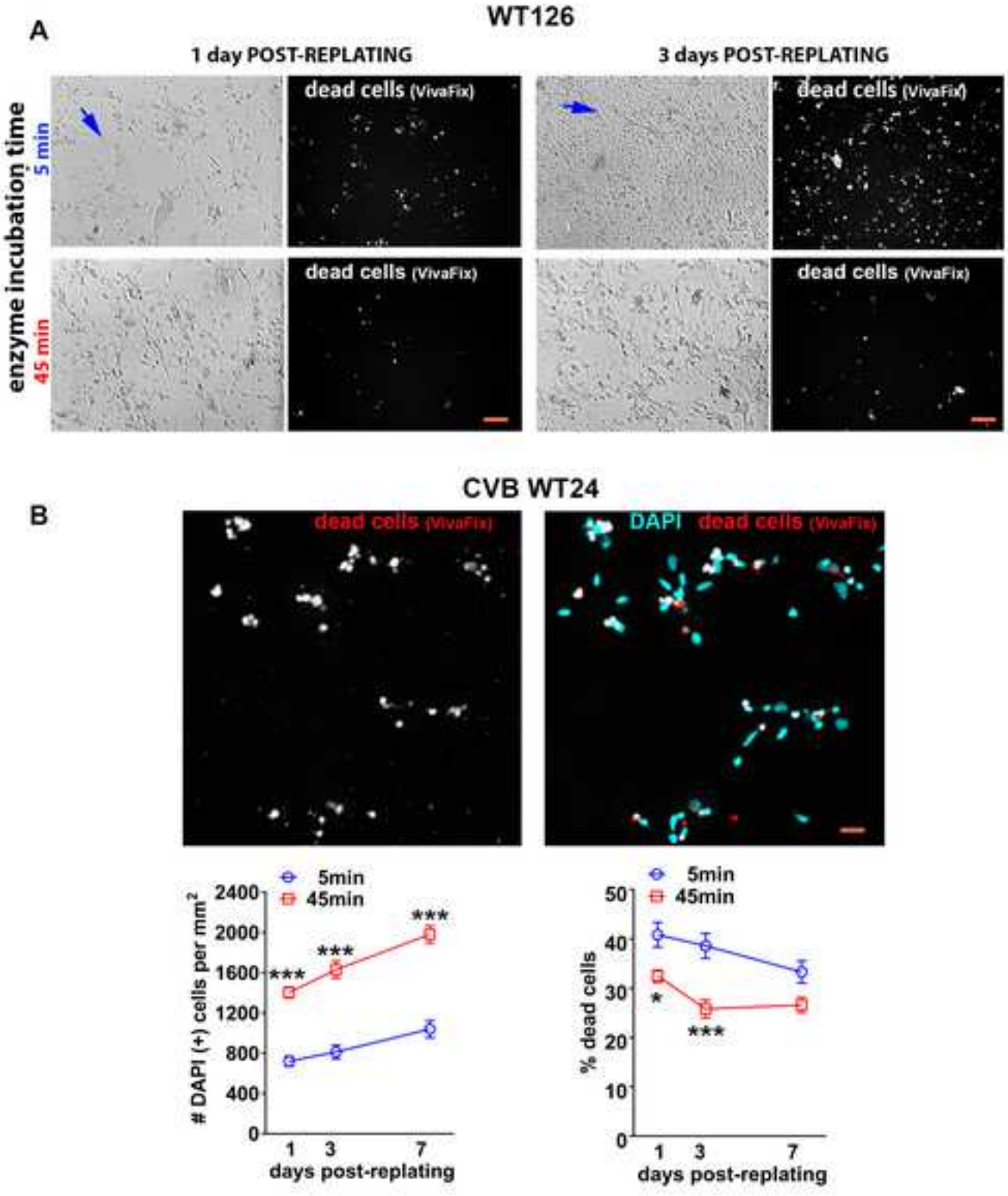

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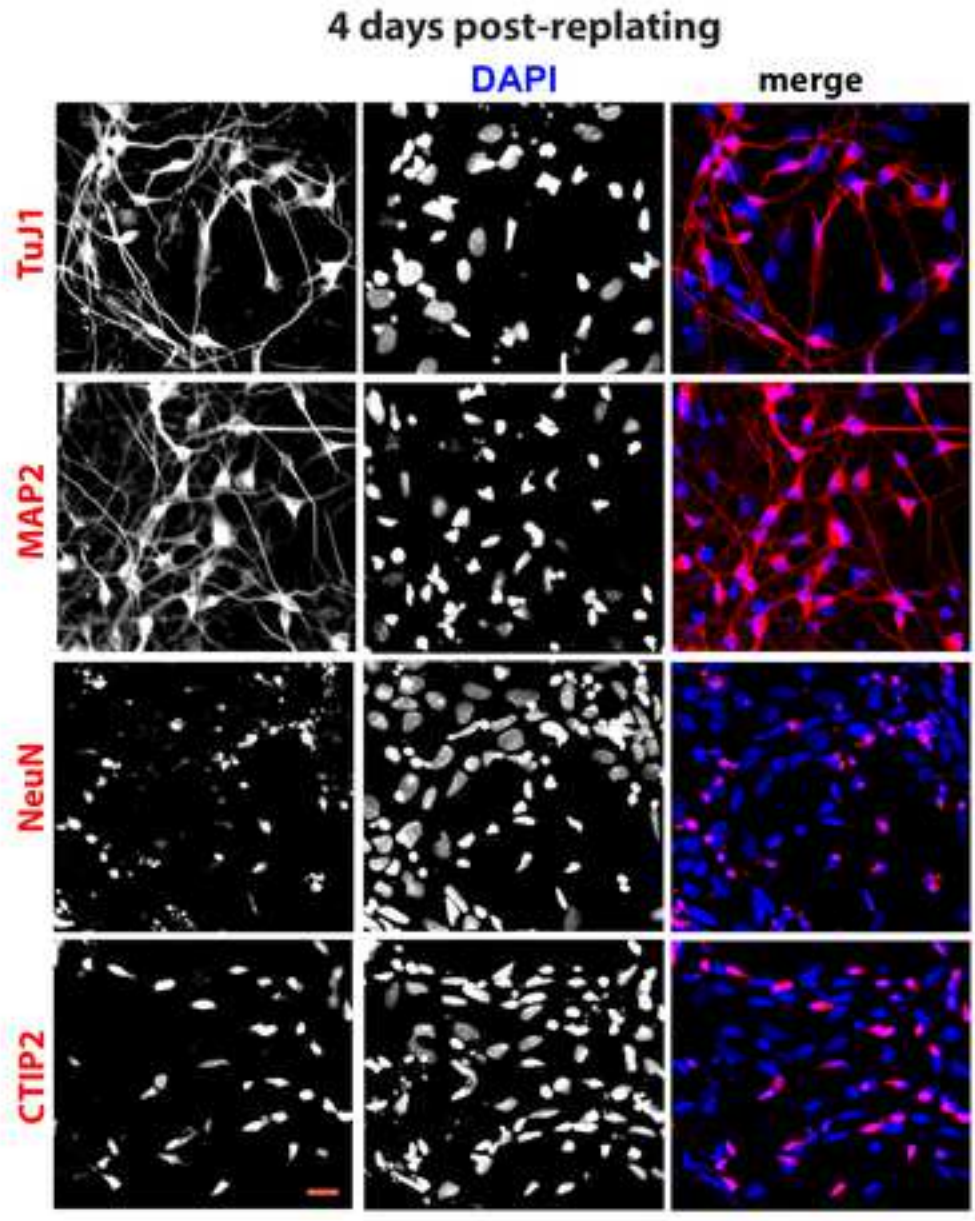

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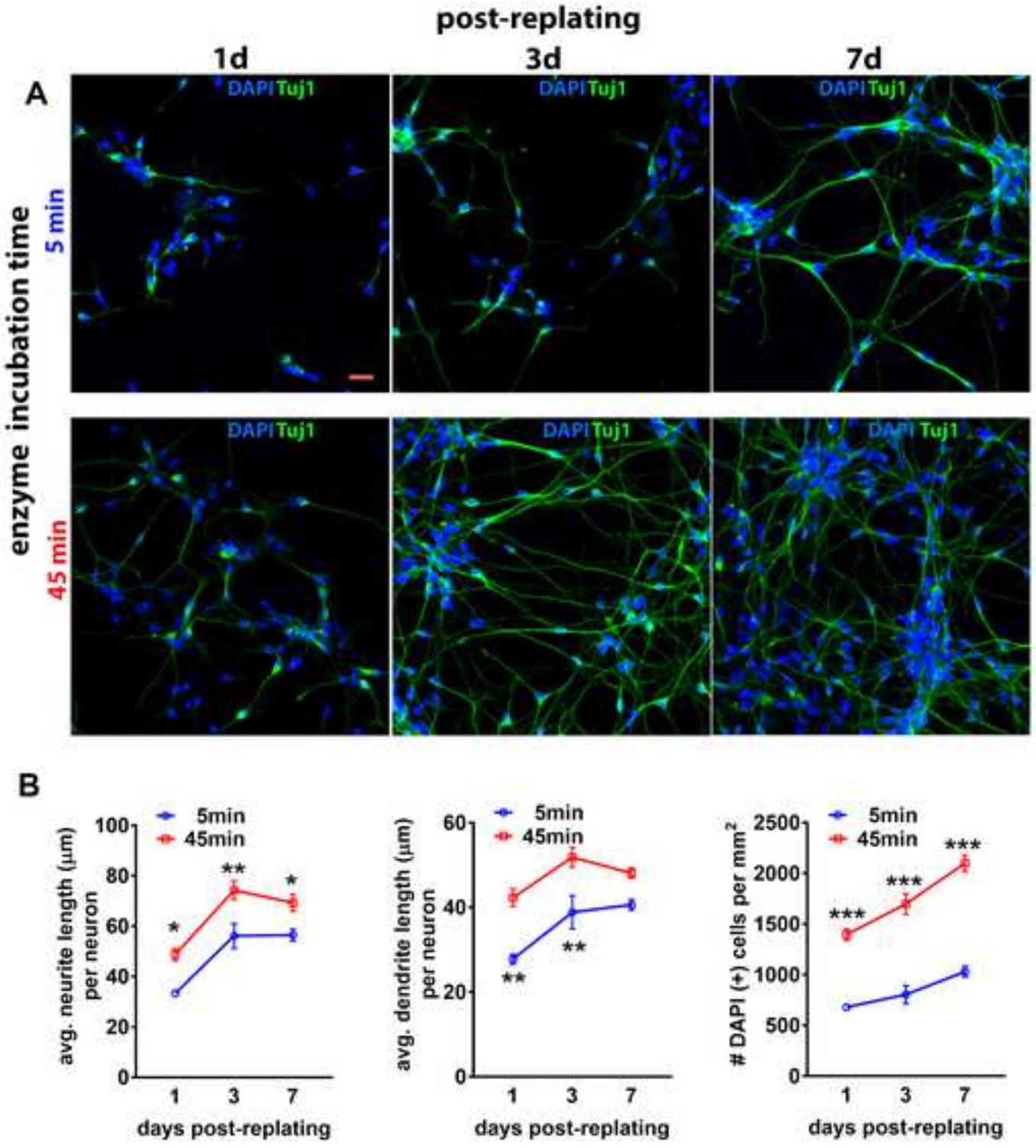

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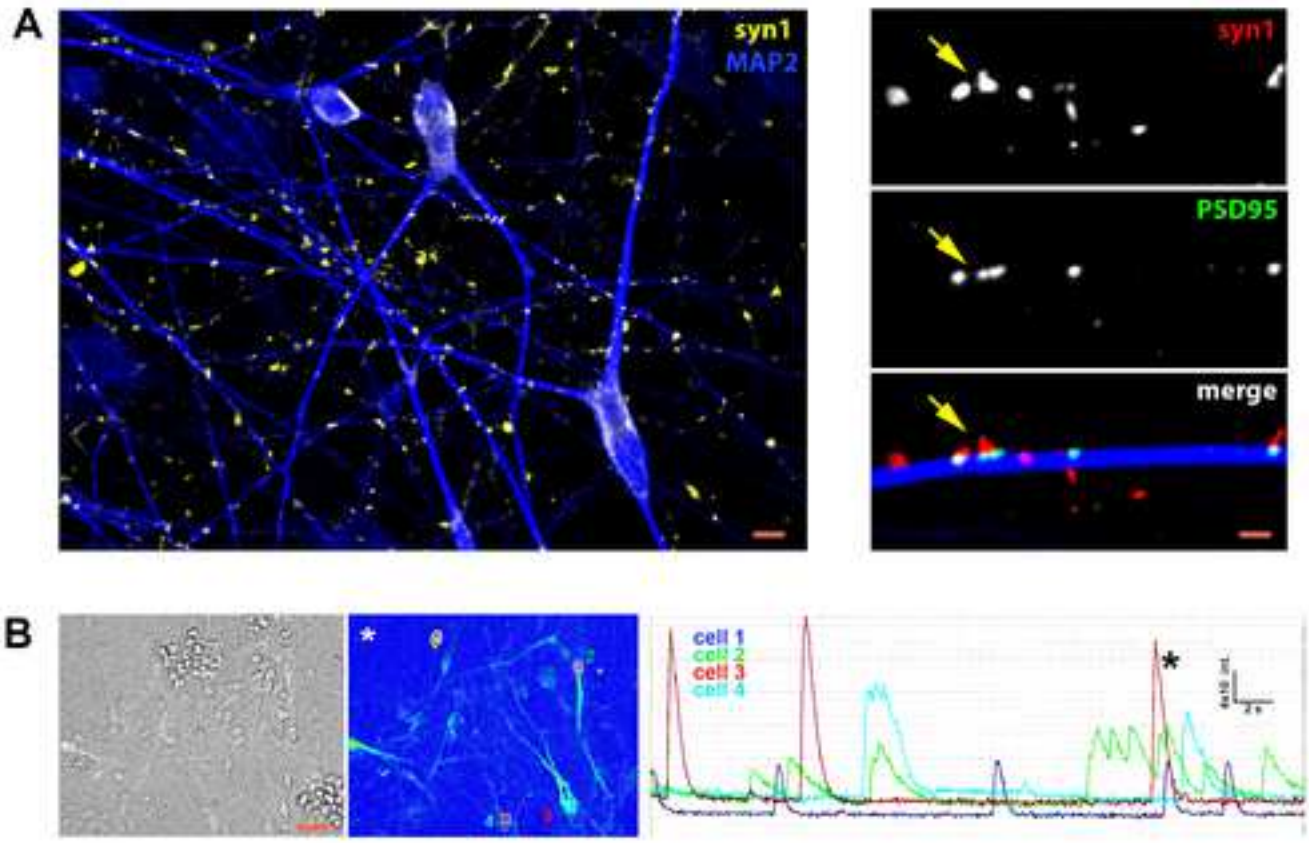

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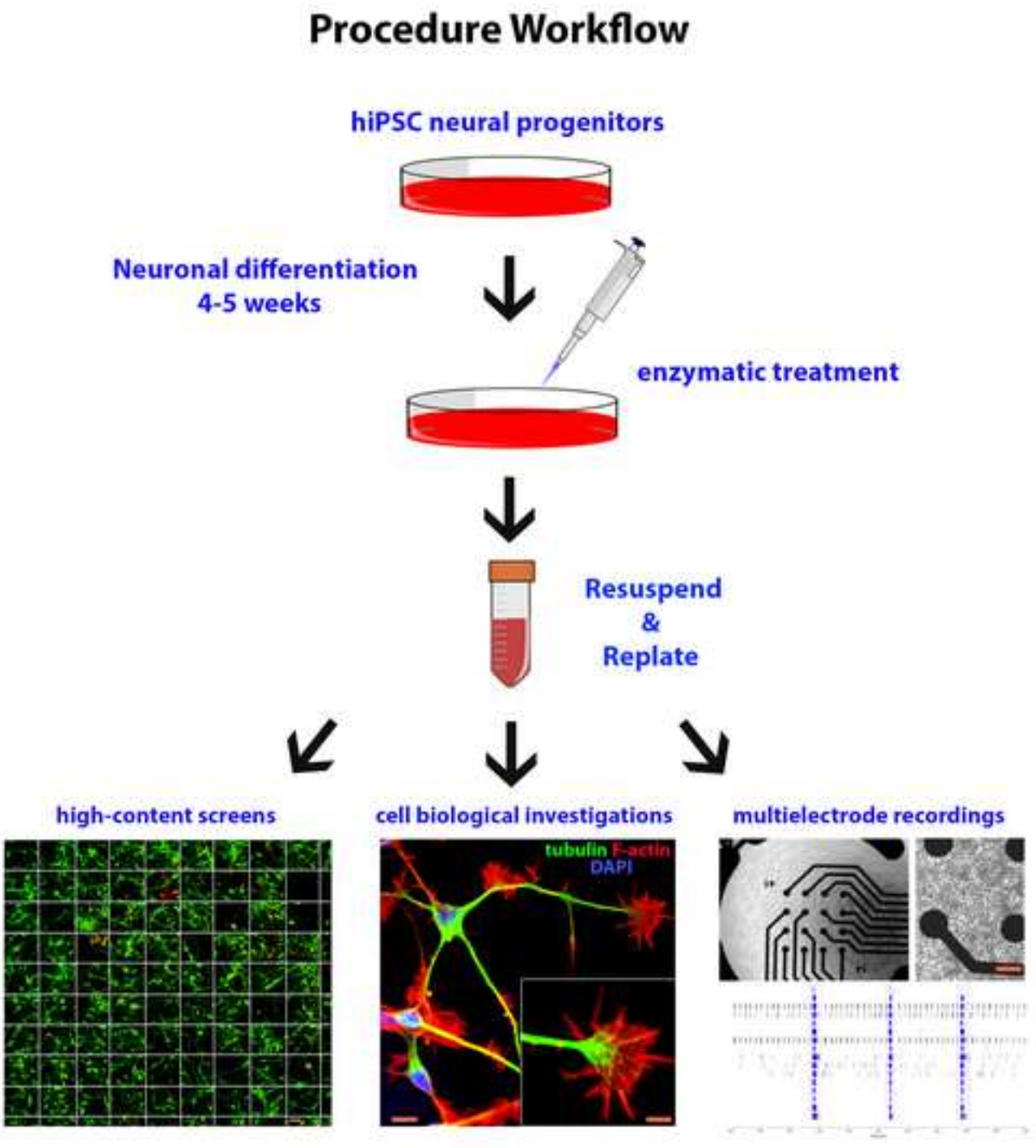

Figure 6 Calabrese et al.

Post Replating Media

Name of Reagent/ Equipment	Company
L-Ascorbic Acid	Sigma
dibutyryl-cAMP	Sigma
Human BDNF	Peprotech
B27 (50X)	Thermofisher Scientific
DMEM/F12 with Glutamax	Thermofisher Scientific
Human GDNF	Peprotech
Glutamax	Thermofisher Scientific
Mouse Laminin	Sigma
MEM Nonessential Amino Acids	Thermofisher Scientific
N2 (100X) Supplement	Life Technologies
Neurobasal A Media	Thermofisher Scientific
Neurobasal Media	Thermofisher Scientific
SM1 Supplement	StemCell Technologies
sodium bicarbonate	Thermofisher Scientific

Plate Preparation

Name of Reagent/ Equipment	Company
10cm Tissue Culture Dishes	Fisher Scientific
6-well Tissue Culture Dishes	Thomas Scientific
Mouse Laminin	Life Technologies
Poly-Ornithine	Sigma
UltraPure Distilled Water	Life Technologies

Replating Reagents

Name of Reagent/ Equipment	Company
100mM Cell Strainer	Corning
384-well plate, uncoated	PerkinElmer
DPBS	Life Technologies
Poly-D-Lysine-Precoated 384-well Plates	PerkinElmer
StemPro Accutase	Life Technologies

Fixation Materials

Name of Reagent/ Equipment	Company
37% Formaldehyde	Fisher Scientific
Sucrose	Fisher Scientific

Immunostaining Materials

Name of Reagent/ Equipment	Company
Alexa Fluor 488 Goat anti-mouse	Invitrogen
Alexa Fluor 568 Goat anti-chicken	Invitrogen
Alexa Fluor 647 Goat anti-chicken	Invitrogen
Alexa Fluor 561 Goat anti-rat	Invitrogen

DAPI	Biotium
mouse antibody against b3-tubulin (TuJ-1)	Neuromics
rat antibody against CTIP2	Abcam
chicken antibody against MAP2	LifeSpan Biosciences
chicken antibody against NeuN	Millipore
rabbit antibody against MAP2	Shelley Halpain
mouse antibody against PSD-95	Sigma
rabbit antibody against Synapsin 1	Millipore
Bovine serum albumin (BSA)	GE Healthcare Life Sciences
Titon X-100	Sigma

Viability Markers

Name of Reagent/ Equipment	**Company**
Vivafix 649/660	Biorad

Calcium Imaging

Name of Reagent/ Equipment	**Company**
AAV8-syn-jGCAMP7f-WPRE	THE SALK INSTITUTE, GT3 Core Facility

hiPSC-derived NPCs

WT 126 (Y2610)	Gage lab
CVB WT24	Yeo and Goldstein labs

Catalog Number	Comments/Description
A4403	Add 1ml of 200mM stock to 1L of N2B27 media
D0627	Add 1 µM
450-02	10 ng/ml final concentration
17504044	Add 20 ml to 1L N2B27 media
31331093	Add N2 and distribute in 50 mL conicals; parafilm wrap lids
450-10	10 ng/ml final concentration
35050038	Add 10 ml to 1L N2B27 media; glutamine supplement
P3655-10mg	Add 100 µl to 50 mL N2B27
11140035	Add 5ml to 1L N2B27 media
17502048	Add 5ml to 500mL media
10888022	Combine with DMEM/F12 to generate N2B27 media for CVB wt cells; neural basal A
21103049	for WT126 cells; neural basal media
5711	Add 1:50 to media
25080-094	Add 10ml to 1L N2B27 media

Catalog Number	
08772-E	Plastic TC-treated dishes
1194Y80	NEST plates
23017-015	Add 1:400 on plastic
P3655-10mg	Add 1:1000 on plastic
10977-015	To dilute Poly-L-Ornithine

Catalog Number	Comments/Description
431752	Sterile, individually wrapped
6007550	Coat with PLO and Laminin
14190144	Dulbecco's phosphate-buffered saline
6057500	Rinse before coating with laminin
A1110501	Apply 1mL/10cm plate for 30-45 minutes; proteolytic enzyme

Catalog Number	Comments/Description
F79-1	Dissolved in PBS
S5-12	0.8 g per 10 ml of fixative

Catalog Number	Comments/Description
A-11001	secondary antibody
A-11041	secondary antibody
A-21449	secondary antibody
A-11077	secondary antibody

40043	visualizes DNA
MO15013	early stage neuronal marker
ab18465	layer 5/6 cortical neurons
LS-B290	early stage neuronal marker
ABN91	late stage neuronal marker
N/A	early stage neuronal marker
P-246	post-synaptic marker
AB1543	pre-synaptic marker
SH30574.02	10% in PBS for blocking
9002931	Dilute to 0.2% on PBS for permeabilization

Catalog Number	**Comments/Description**
135-1118	cell death marker

Catalog Number	**Comments/Description**
N/A	calcium reporter in a viral delivery system

N/A	Marchetto et al., 2010
N/A	unpublished

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1. Please provide full names for all authors.

The authors are now listed with their full name in the manuscript.

2. Additional details are needed. Please note that we need a specific protocol throughout and not a generalized protocol.

We have further edited the protocol.

1.1: Please provide a citation for the differentiation.

Done (line 151)

1.2: What media is used?

Although the type of media is not relevant to the success of the replating procedure the media used in this paper are now listed in section 1.2

2.1: What volume and concentration is used? Please provide these throughout.

Done.

***Note*: In section 1.2, volume is provided whenever concentration is not available from supplier.**

3. Please remove commercial names from the protocol as much as possible: Accutase, VivaFix, etc.

We have tried to comply as much as possible.

4. Please shorten the figure legends and move some of this text to the Representative Results.

We have tried to comply wherever clarity wouldn't be affected by shortening the text.

5. Please do not abbreviate journal titles in the References.

Journal titles are now listed in full length.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors response addresses all the previously raised concerns.

Minor Concerns:

Optional - Obtaining an even distribution of neurons in the wells can be important for assays. Apart from using a figure of eight motion to deliver the cells into a well, do the authors have any other pointers to obtain a homogeneous distribution?

We have now included in the revised manuscript the statement that adding laminin to the replating medium can also improve the uniformity of cell distribution. In addition, automated liquid handling devices might yield improved uniformity (line 270-274) .

Reviewer #2:

Overall, the authors did a fair job of addressing our comments and the article is improved. However, its still unclear what the advantage is of their methods are and why the authors think they are more well- suited for high throughput approaches. Nor have the authors demonstrated convincingly that their methods show better survival or function for re-plating of neurons than simple dissociation methods. Comments to the specific details are found below in red.

It is difficult to understand why the reviewer continues to be unclear on the advantage of the described method. Previous versions of the manuscript repeatedly demonstrated an approximate doubling of recovery and survival of replated cells using the extended protease incubation time (a simple and minor modification of standard procedures). Moreover, we used multiple assays to demonstrate morphological and functional viability of the neuronal cultures. It is our belief that the community of investigators using iPSC-derived neurons would find this 2-fold viability improvement very useful. If Reviewer 2 chooses to disbelieve our

documented results, or to believe that lower viability is somehow desirable, there appears to be little else we can do to satisfy this individual.

We ask the Editor to consider that, regardless of whether there is advantage to the extended protease incubation (there clearly is), the most important goal of the paper is not to argue that all investigators must immediately switch to using our superior method, but rather to share widely amongst the research community a straight-forward protocol for replating monolayer cultures of iPSC neurons in a manner that can facilitate high content screening applications.

Reviewer #2:
Calabrese et al., proposes a methods protocol for the dissociation of neurons that have been differentiated from iPSCs. The authors report that this method is optimized for high throughput analysis of neuritogenesis and synaptiogeneis, but the authors fail to provide any results that back up these statements. Further, the work they show is sloppy, incomplete and lacks any sort of quantification, statistical methods or scientific rigor. For this reason, we do not recommend this manuscript for publication in Jove.
AUTHORS: The reviewer appears to misunderstand the objective and publication criteria for a JoVE article, and to be unfamiliar with the history of the numerous JoVE articles that contain minimal quantitative data. In addition, several comments indicate the reviewer may have failed to carefully read the manuscript, thereby overlooking statements that were actually included. Moreover, this review supplies very little constructive criticism that would guide the improvement of the manuscript, nor cited papers or examples from the established scientific literature that support the criticisms. Thus, the aggressive tone and and unsupported accusation that our paper "lacks…scientific rigor" is unprofessional and unhelpful. Nevertheless, we have tried to address below those comments that are relevant to improving the quality and clarity of the manuscript. We have added several new experiments, quantified additional features, and included additional sample images in the revised manuscript.
While Jove articles may have different publication criteria than other journals, It is of the reviewer's opinion that basic empirical methods that use quantification and statistical measures should be the premise for any article that merits publication in a scientific journal. Below we list specific comments for the authors to consider.

We agree that acceptable publication standards include reproducibility. For many methods articles in JoVE qualitative descriptions have commonly been used. Nevertheless, we complied with the reviewer's suggestion to include quantification of key results, which demonstrated a clearly robust increase in cell viability for extended protease incubation. As shown in the previous submission, there was no overlap in the

data points for 5 min vs 45 min Accutase. We have now reported statistical analyses, as suggested by the reviewer (Figures 1, 2 and 4), and these fully support our original assertions.

Comments to Figure 1:
* For the VivaFix and calcein-AM stains (Fig 1C), it appears that the stains overlap for a subset of the cells which is confusing as these are labeling dead/live cells respectively. Further, the calcein-AM does not appear to be selectively labeling live cells as the cells that are in the brightfield images that have distinct morphology with process are not labeled by this dye. Therefore, the use of these dyes to distinguish between live and dead cells does not warrant validity and should not be used to quantify neuronal viability. The authors should use other live / dead stains which are more specific.
AUTHORS: These statements are demonstrably incorrect. We carefully reexamined the images submitted in Figure 1 from our original manuscript and found no cells present in the brightfield image that lacked either calcein or VivaFix signal. Possibly the reviewer was confused by observing the fainter calcein signal in the population of flatter cells. We found only rare instances of cells that showed a faint VivaFix signal while also exhibiting calcein signal. VivaFix is an early cell death marker (because it detects loss of membrane integrity), so occasionally a cell might take up both calcein-AM labeling and VivaFix while it is in the process of dying.
To improve the clarity of these experiments we have moved the VivaFix experiments to the new Figure 2, have added quantification, and provided sample images that strengthen clarity of the assay results. We removed the calcein staining altogether from the new samples, as it was originally included only as a means to identify neuronal processes. All data support the original statements regarding the improved viability of neurons when using the "extended Accutase protocol" that we recommend.

Mostly acceptable. However, the number cells scored should be listed for this figure.

We have now stated in the revised legend to Figure 2 that we scored a minimum of 1500 cells per condition (line 440).

* Upon comparing images from the first day versus third day post- re-plating of cells that have accutase treatment for 45 minutes appear to be loosing their process and dying as compared to day 1. While they do look healthier than the 5 minute accuatase- treated cells they still look like they are significantly comprised. It is unclear what these cells look like at

later time points? The authors should investigate the health of the cells and morphology of outgrowth in a much more rigorous fashion. For example, the authors could show time-lapse images of the same cells to show how they grow and change along with quantification of cell length and soma size.

AUTHORS: The images at day 1 and day 3 did not belong to the same field, and indeed because these are fixed samples they are from different specimens. We are unclear on the basis for the reviewer's claim that the cells "appear to be losing [sic] their processes", as no specific evidence was cited.

In the revised submission we have added multiple experiments, quantitative analyses, and numerous examples that rigorously establish the viability, healthy neurite morphology, and electrical activity of the replated cultures (see new Figures 1-6).

Mostly acceptable. However, a few comments;

1) The authors emphasize that the neurons will detach as a "neuronal sheet" (Figure 1 and lines 208-210) however, they cell suspension is filtered through a 100uM filter. Its unclear how cells in a "sheet" like structure pass through this filter as this size of filter will likely only allow very small networks of neurons or even single large neurons through. The authors should clarify how many cells they expect to pass through this sized of a filter if the cells are not dissociated but in a "sheet".

The reviewer still does not appear to understand the protocol, or its biological rationale. As clearly stated in the protocol the proteolytically treated cell suspension is filtered <u>after</u> trituration. As also stated in both previous versions of the manuscript, the dense meshwork of neurite-bearing cells lifts off as a sheet during the first ~5 min of protease incubation, and we then wait while the cultures continue to incubate in protease for an additional 40 min <u>before performing the trituration step,</u> which dissociates the sheet of cells into a suspension of individual cells used for replating. These steps and their rationale are described in three places in the manuscript –the introduction (lines 101-109 and 112 and 116), the protocol (step 3.6 and 3.7 from line 225 to line 232), results (from line 337 to 373), and Figure 1 legend (line 420). We do NOT pass the sheet of cells through a filter. The <u>cell suspension that results after trituration</u>, <u>*not the sheet of cells*</u>, is passed through the 100um filter. The biological rationale is that the extended proteolysis of the sheet of cells minimizes damage to neurons during the subsequent trituration. Trituration is a mechanical disruption of the cell meshwork. It is one of the steps most subject to user-specific subjectivity and variability in neuronal culture. When neuronal cultures form a dense meshwork, the "vigor" of trituration needed to yield a robust suspension of individual neurons can be quite high. We discovered that additional protease incubation time promotes the use of gentler mechanical trituration. Several of our colleagues have adopted our method, and report improved success in iPSC-derived neuronal viability.

2) For the micrographs in Figure 4, the neurons that were exposed to enzyme for 5 minutes plated 1-7 days looks just as healthy, simply just less dense than the cells exposed to accutase for 45 minutes. Did the authors subject the cells that were exposed to the 5 minute enzyme treatment to the same exact plating methods as the cells subjected to 45 minute enzyme treatment?

We agree that the neurons that have survived replating under either conditions are mostly healthy, and that neurites regenerate in both. However, the graphs in Figure 4 clearly demonstrate that neurite outgrowth on a per-cell basis shows a modest increase for the extended protease incubation protocol, implying that surviving cells pay a "cost" when the protease incubation is too short. Statistical analyses bear this out, and have been included in the revised manuscript. Thus, these quantitative data reveal that the extended protease incubation is superior, as judged by functional measures beyond mere cell survival.

3) For the neurite quantification, how did the authors distinguish between processes belonging to different neurons? The cells are so dense that its seems determining which process belongs to which neuron is almost impossible at the later time points.

We did not attempt to distinguish among processes belonging to individual neurons, for reasons the Reviewer stated. Instead, we quantified the total length of neurites (or dendrites) per field-of-view, then divided by the number of neuronal cell bodies per field to yield a value for average neurite length per neuron. This is a standard operating procedure in many high content screens of neuritogenesis.

* The authors claim that their methods encourage neurite regeneration does not seem accurate. When comparing images from Post-replating day 1 to day 3, it actually looks like at the later time points the neurites are actually degenerating.
AUTHORS: Nowhere did we claim that these methods "encourage" neurite regeneration. Instead, we state that replated neurons spontaneously regenerate neurites, which are naturally sheared off by the trituration process, and that this affords an opportunity to study the neurite initiation and growth cone biology in uncrowded culture conditions. Please see the response to Reviewer 1 above, where we discuss that this shearing of neurites occurs regardless of whether the Accutase incubation is short or long.
We do not understand or agree with the reviewer's unexplained assertion that the neurites at day 3 are degenerating.

In most cases, neurons that are re-plated after enzyme disassociation regenerate neurites. Its unclear what the advantage is to the authors protocol other than perhaps unremarkable differences in cell death. Although, I am not convinced this is true given the lack of details in the manuscript to comparing a 5 - minute enzyme dissociation method to their methods.

Again, and as stated above, the advantage is that cell survival after replating is doubled (this finding is statistically significant). The differences seen in the cell death marker assays are also statistically significant, although the magnitude of the differences are more modest. Please keep in mind that this type of assay for cell death, where only the cells that are adhered to the culture substrate are stained, will inevitably underestimate the true degree of cell death, since some of the dead cells detach from the substrate and end up floating in the culture medium. The doubling of the viable cell population as seen by DAPI quantification is probably a more accurate depiction of the potent difference in cell viability between the short and long protease incubation times. We included the cell death assay results here as an alternative means to demonstrate the superiority of the prolonged protease incubation.

* The authors suggest that other proteases can work with their methods however the manuscript only shows data from accutase as stated " In the protocol described below, neurons are incubated for 40-45 min with a mild protease, such as accutase". If the authors to make the claim that they have used other dissociation buffers they need to show the data.
AUTHORS: We did not state that we have used other proteases, and only Accutase is referenced in this manuscript. To be clearer we have removed the words "such as". However, it seems likely that other mild protease products might also work well with these methods and would also benefit from a longer incubation period that loosens up the meshwork of neurites prior to trituration.
Acceptable
* The methods of adding dissociation buffer are not clear. The authors state they add 1 ml of accutase to a 10 cm plate. How do the authors distribute the accutase evenly through the well is not clear as this volume is much too low to cover the surface area of 80 cm2. In addition, the authors do not state how confluent or mature the cells should be before dissociating.
AUTHORS: 1ml trypsin (another proteolytic enzyme) is commonly used on 10 cm plates to split cell lines in labs across the world. A film of proteolytic solution is usually enough. Thus, we disagree with the reviewer's assertion that this volume is too low for a 10 cm plate. Adding more volume is fine, but usually this would only be necessary in environments with

extreme low humidity, and we now add this caution in the revised manuscript (lines 152-154).
Regarding the maturation state of the cultures before dissociating, it was clearly stated in the protocol that we replate NPC-derived neurons at 4-5 weeks post-differentiation. This is now repeated in multiple places so the information does not get overlooked (line 120 protocol; line 351 and 418 figure legends).
Regarding plating density, the manuscript clearly stated the replating density of neurons for both 384-well plates and 24-well plates "Plate ~10,000 cells/well for a 384-well plate; it is recommended to plate ~150,000-200,000 cells/well for a 24-well plate to maximize survival rate".
Regarding the confluency just prior to dissociation, we had originally stated the cultures should be sufficiently confluent to form a neuronal network with densely populated processes throughout the culture upon differentiation. However, in the revised protocol we now specify 80% confluency.
Acceptable
* The authors comment (page 6 first paragraph) the peculiar and confusing observation that the cells survive better when exposed to accutase for 45 minutes compared to 5 minutes. However, the authors provide no basis or rationale for this curious finding should? Presumably they are using less enzyme or??
AUTHORS: We believe the explanation we provided in the original manuscript was clear, and logical. For replating large diameter cultures the "extended Accutase protocol" quite clearly improves the viability of the cells (now demonstrated quantitatively for a second line of hiPSC). We previously acknowledged that this observation seems initially counterintuitive. However, it makes sense when one considers that the protease incubation detaches the neuronal meshwork as a large sheet (because of the dense and highly interconnected neurites), and that any harsh or lengthy trituration applied to break up this sheet into a cell suspension could cause extensive membrane damage, not only shearing the neurites but also damaging the cell bodies.
Since this rationale was spelled out in the original manuscript, we are puzzled by the reviewer's assertion. We assume the revised manuscript, with its attempt at greater clarity, will dispel any concerns.
As noted above, if the cells are lifted off in sheets but then they are filtered through a 100 uM filter which only allows small networks of cell through its unclear how the "neuronal meshwork" is maintained and thus viability improved.

As stated above and repeatedly in the manuscript, we do NOT filter the sheet of cells, but rather triturate them to release the cells into suspension, and only THEN do we pass the

sample through the filter. We presume that the neurons do not maintain their network connections during this procedure.

Figure 2.
* The authors claim that they can detect late- stage neuronal markers immediately after re-plating as shown on the right-hand side of the panel. However, its entirely unclear what the replating does to the cells at right after and later stages of the differentiation. The authors should show investigate the composition of the markers before disassociating to compare with the post one day plating. Further, as described above, the authors should examine markers thereafter.
AUTHORS: The revised submission contains several additions that confirm the viability and differentiation state of the neurons.
Acceptable
* The authors fail to demonstrate any quantification or statistical analysis of makers by ICC or Q-RT-PCR for these methods. Further, there is no information on the experimental n or what the variability across differentiations and different cell lines. At a very minimum, the authors should base and list their conclusions based on fundamental scientific reporting.
AUTHORS: This is a methods video paper, not a primary research or discovery paper. Nevertheless, we have included several additional quantitative results in the new manuscript, and included the sample size and statistical reports as appropriate. It was unclear to us what the reviewer meant by investigating "the composition of the markers." It is our intention to share a straightforward replating method with the JoVEaudience, and the investigation of marker time courses is beyond the scope of this paper.
It is still unclear what the advantage is of their methods. The only difference that they are claiming is that there is increased viability however, the data they show does not warrant that claim. The graph in Figure 2 shows that the % number of dead cells post-plating from their method is anywhere from 5- 15% more (a ~1.2 fold difference is unremarkable) than the 5- minute accustase-treated cells. As the authors are reporting the SEM, its unclear if this is even significantly different. The same is true for the # of live cells per mms. At best there appears no more than a 2-fold increase in the cell density after re-plating and its also unclear if this is significant.

As stated above, the 2-fold increase in cell density, which is maintained over several days, is statistically significant. The more modest decrease in the magnitude of cell death is addressed above. There is also a statistically significant increase in neurite outgrowth. We believe these observations are meaningful to researchers who desire to optimize the efficiency and viability of their culture method.

* The staining of markers for NeuN and CTIP2 is clearly incorrect. These proteins are transcription factors and are found in the nucleus of cells. Antibodies directed against these proteins show clear distinct staining only in the nucleus, thus the staining shown here is not specific. Thus, the claim that the authors show late stage neuronal markers immediately after replating is not valid. The authors should use different antibodies and check for accurate staining patterns.
AUTHORS: In the revised submission we have included new images and adjusted the display settings for CTIP2 and NeuN so that it is clear that their greatest localization is nuclear (as it was previously). Many transcription factors have biologically relevant localizations outside the nucleus, even though they are usually most concentrated in the nucleus. We have confirmed the specificity of these antibodies using immunoblots and observed similar staining patterns in cultures of rodent primary neurons.
Acceptable- these stains looks correct in contrast to the stains the authors showed previously.

Figures 3 and 4
* The authors make the claim that their methods allow the study of synapse assembly and maturation but in their results the only data they have to back this statement is an image of 1 neuron that shows co-localization of PSD95 and synapsin while the image in Figure 4 shows virtually no co- localization of these markers. Therefore, its entirely unclear how many functional synapses are observed in these cultures. The authors should evaluate and quantify many more neurons and or demonstrate more convincing results that these cultures contain synapses and are electrophysical active if they wish to make this claim.
AUTHORS: We have included quantification of synapses, calcium imaging, and additional examples of synapse distribution in the revised submission.
Acceptable

* The authors state that a this method is compatible for examining growth cone initiation after replating but it unclear why this is any better than other methods. And again, there is no quantification of growth or measurements of the growth cones.
AUTHORS: Detailed evaluation of growth cones is not the point of this paper, and we mention it only as one of many examples of how replated neurons can be used. Figure 4 (new Figure 6) was intended only to summarize the protocol work flow and provide a few examples of application. We now state in the revised paper that it is advantageous for certain types of imaging-based experiments to study growth cones in a cellular environment where the growth cone is not densely surrounded by other cellular material, because it

facilitates high resolution imaging. Optimal imaging is not readily achievable during the differentiation of neurons from progenitor cells in either 2D or 3D culture (because the cultures tend to be dense), although it certainly could be done using advanced technology.
Acceptable

General Comments
* For all Figures, its unclear how many times of each of the data sets were repeated, how many and the name of the iPCS cells they used, nor any quantification of any sort. This manuscript entirely lack basic scientific methodology and principle.
AUTHORS: We have included this information in the revised submission.
* The micro graphs do not show scale bars.
AUTHORS: The original version of the manuscript specified the image width in the Figure Legends for each figure. Because the reviewer clearly overlooked this, we have now included scale bars in all images.
* With regard to "lines 54-63" there is no solid evidence for claims of non-beneficial effects of dividing NPCs in cell cultures if there is such then the author should cite a proper publication that highlights these issues.
AUTHORS: We have not stated that dividing cells in culture are detrimental per se. Our text clearly stated that continuously dividing cells can overgrow the culture, competing with neurons for nutrients and "space". This in itself is not a problem for short duration culture, but becomes an issue when cultures are maintained for many weeks without replating. For cells, like neurons, that are sensitive to nutrient depletion, it is important to maintain robust metabolism. It is the central reason why cell lines (HeLa, 3T3, CHO, etc) are "passaged" (i.e., replated) to maintain cultures over long periods.

* With regard to "lines 74-75", the authors should clarify what the normal range of incubation time is and cite the source.
AUTHORS: We included the "typical" range of 3-10 minutes, and cited more than one reference in the original manuscript.
* The paper does not specify working cell density for all their results which can play a role in their incubation times.
AUTHORS: Cell density was stated in the original manuscript.
* With regard to lines 1"72-173", recommendation is not based on any sort of comparison study by authors or elsewhere. Authors should cite the source.
AUTHORS: The recommendation is based on our experience.