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Generation of Neurospheres from Mixed Primary Hippocampal and Cortical Neurons isolated from E14-E16 Sprague Dawley Rat Embryo

--Manuscript Draft--

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22nd May, 2019

Dear Editor,

Hereby, we submit our revised manuscript (JoVE59800R1) entitled **" Generation of Neurospheres from Mixed Primary Hippocampal and Cortical Neurons isolated from E14-E16 Sprague Dawley Rat Embryo "** for publication in ***Journal of Visualized Experiments (JoVE)***.

For this revision of the manuscript, we have performed all the revisions asked the editors both in the main text and in video.Below we have provided a point by point response/rebuttal to all the comments raised. We have highlighted all the changes in red color for everyone to track.

Therefore, we urge you to kindly consider this manuscript as a very important advancement and consider for publication in your esteemed journal. We sincerely look forward to receive a favourable decision on this manuscript.

Best regards.
Sincerely,
Surajit Ghosh (Corresponding author)

REBUTTAL

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain the same.

Reply: We want to thank the editorial team for their hardwork in formatting the manuscript so nicely and we have retained the same style.

2. Please address specific comments marked in the manuscript.

Reply: We are thankful to the editors for their specific and insightful comments. We have indeed addressed all the specific comments in the manuscript and have highlighted them in red color. All these changes can be easily tracked in the highlighted manuscript. However, we do not agree with one or two changes that have been suggested by the editors and hence have retained the original style. One example is that the editors have asked us to combine section 3 and 4 in the protocol section as 2.1 and 2.2. However, we feel they are best under separate sections. We hope the editors will respect our point of view. The rest of the changes have been mostly made.

3. Once done, please proofread the manuscript for any grammar or spelling issues.

Reply: We did proof read the manuscript for grammatical and spelling errors and hope the manuscript is now mostly without errors.

Video:

1. 2:40: Foetus should be changed to fetus.

Reply: We have made this change in the video.

2. 5:12: Please use the degree symbol and leave a single space between the number and unit. Also, for minutes please use the abbreviation min. Please perform the change throughout the video.

Reply: We have made this change in the video now.

3. 6:13: Please reformat to ~2.51 x 107 cells/mL

Reply: We have reformatted it in the new video.

4. 7:05, 8:24: Please leave a single space between 200 µm and 20 µm.
   Reply: We have made this correction and it is now reflected in the new video.
5. Please include all the result figures both in the text and the video in the representative result section. For example, few figures are not shown in the text (8:57) and figures 6-10 are not shown in the video.

Reply: We have included all the figures from (6-10) in the video now along with narration and the figure (8:57) has been included in the text as figure 4. It can be tracked in the highlighted manuscript marked red.

6. Once done, please ensure that the video is no more than 15 min and contains 59800R2 in the name.

Reply: The new video is less than 13 min and contains 59800R2 in the name.

7. Production comment: There is still one jump cut 4:12 that should be replaced with a crossfade.

Reply: The jump cut at 4:12 has been replaced with crossfade. .

TITLE:

Generation of Neurospheres from Mixed Primary Hippocampal and Cortical Neurons Isolated from E14-E16 Sprague Dawley Rat Embryo

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KEYWORDS:

neurospheres, primary neuron culture, hippocampal neurons, cortical neurons, mixed culture, Sprague Dawley, embryo, neural progenitor cells, NPCs

SUMMARY:

Presented here is a protocol for the spontaneous generation of neurospheres enriched in neural progenitor cells from high density plated neurons. During the same experiment, when neurons are plated at a lower density, the protocol also results in prolonged primary rat neuron cultures.

ABSTRACT:

Primary neuron culture is an essential technique in the field of neuroscience. To gain deeper mechanistic insights into the brain, it is essential to have a robust in vitro model that can be exploited for various neurobiology studies. Though primary neuron cultures (i.e., long-term hippocampal cultures) have provided scientists with models, it does not yet represent the complexity of brain network completely. In the wake of these limitations, a new model has emerged using neurospheres, which bears a closer resemblance to the brain tissue. The present protocol describes the plating of high and low densities of mixed cortical and hippocampal neurons isolated from the embryo of embryonic day 14–16 Sprague Dawley rats. This allows for the generation of neurospheres and long-term primary neuron culture as two independent platforms to conduct further studies. This process is extremely simple and cost-effective, as it minimizes several steps and reagents previously deemed essential for neuron culture. This is a robust protocol with minimal requirements that can be performed with achievable results and further used for a diversity of studies related to neuroscience.

INTRODUCTION:

The brain is an intricate circuitry of neuronal and non-neuronal cells. For years, scientists have been trying to gain insight into this complex machinery. To do so, neuroscientists initially resorted to various transformed nerve-based cell lines for investigations. However, the inability of these clonal cell lines to form strong synaptic connections and proper axons or dendrites have shifted scientific interest to primary neuron cultures[1,2]. The most exciting aspect of primary neuron culture is that it creates an opportunity to observe and manipulate living neurons[3]. Moreover, it is less complex compared to neural tissue, which makes it an ideal candidate for studying the function and transport of various neuronal proteins. Recently, several developments in the fields of microscopy, genomics, and proteomics have generated new opportunities for neuroscientists to exploit neuron cultures[4].

Primary cultures have allowed neuroscientists to explore the molecular mechanisms behind neural development, analyze various neural signaling pathways, and develop a more coherent understanding of synapsis. Though a number of methods have reported cultures from primary neurons (mostly from the hippocampal origin[5-7]), a unified protocol with a chemically defined medium that enables long-term culture of neurons is still needed. However, neurons plated at a low density are most often observed, which do not survive long-term, likely due to the lack of trophic support[8] that is provided by the adjacent neurons and glial cells. Some methods have even suggested co-culturing of the primary neurons with glial cells, wherein the glial cells are used as a feeder layer[9]. However, glial cells pose a lot of problems due to their overgrowth, which sometimes override the neuronal growth[10]. Hence, considering the problems above, a simpler and more cost-effective primary neural culture protocol is required, which can be used by both neurobiologists and neurochemists for investigations.

A primary neuron culture is essentially a form of 2D culture and does not represent the plasticity, spatial integrity, or heterogeneity of the brain. This has given rise to the need for a more believable 3D model called neurospheres[11,12]. Neurospheres present a novel platform to neuroscientists, with a closer resemblance to the real, in vivo brain[13]. Neurospheres are non-adherent 3D clusters of cells that are rich in neural stem cells (NSCs), neural progenitor cells (NPCs), neurons, and astrocytes. They are an excellent source for the isolation of neural stem cells and neural progenitor cells, which can be used to study differentiation into various neuronal and non-neuronal lineages. Again, variability within neurosphere cultures produced using the previously reported protocols presents a barrier to the formulation of a unified neurosphere culture protocol[14].

This manuscript presents a protocol in which it is possible to generate both 2D and 3D platforms by alternating cell plating densities from a mixed cortical and hippocampal culture. It is observed that within 7 days, high-density plated neurons isolated from E14–E16 Sprague Dawley rat embryo free-floating neurospheres are obtained, which upon further culture, form bridges and interconnections through radial glial-like extensions. Similarly, in the low density plated neurons, a primary neuron culture that can be maintained for up to 30 days is obtained

by changing the maintenance medium twice per week.

PROTOCOL:

All experimental procedures involving animal were approved by the Institutional Animal Ethics Committee of CSIR-Indian Institute of Chemical Biology (IICB/AEC/Meeting/Apr/2018/1).

1. Reagent and media preparation

1.1. Poly-D-lysine (PDL) solution: prepare PDL solutions at concentrations of 0.1 mg/mL in deionized water and store in 4 °C until use.

1.2. Dissociation medium: to 1 L of sterile, filtered deionized water, combine the following components in the respective concentrations: sodium chloride (8 mg/mL), potassium chloride (0.4 mg/mL), potassium phosphate monobasic (0.06 mg/mL), D-glucose (1 mg/mL), sodium phosphate dibasic (0.479 mg/mL), and 1 M HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 10 mM]. Vortex all the components to aid proper mixing and store in 4 °C until use.

NOTE: Use the dissociation medium as ice-cold during dissociation but at room temperature (RT) for washing and other purposes.

1.3. Plating medium: plating medium consists of the following: minimum essential medium (MEM) Eagle's with Earle's Balanced Salt Solution (BSS; 88.4%), D-glucose (0.6%), horse serum (10%), and penicillin/streptomycin (1%). Combine the components in the respective ratios and perform the procedure inside a hood under sterile conditions.

NOTE: Always use freshly prepared plating medium to avoid degradation of any component.

1.4. Maintenance medium: prepare the maintenance medium by combining the following in the respective ratios: neurobasal medium (97%), 0.5 mM commercially obtained glutamine sample, B27 serum-free supplement (2%), and penicillin/streptomycin (1%). Combine the components in the respective ratios and perform the procedure inside a hood under sterile conditions. Ensure that all components are freshly prepared.

2. Preparation of coverslips

2.1 Take a 12 mm diameter round glass coverslip and soak it in 1 M hydrochloric acid (HCl) for 4 h.

2.2 Transfer the coverslips in distilled water using a pair of forceps and swirl it gently to get rid of the acid completely.

2.3 Transfer the washed coverslips for an additional round of cleaning in a beaker containing

100% ethanol.

2.4 Before using the coverslips, dry them well in the laminar hood by keeping them on tissue paper.

3. Preparation of poly-D-lysine coated plates for neuron culture

3.1 Take two 24 well plates: one for high density plating and another for low density plating. Open the sterile packets only inside the laminar hood.

3.2 Transfer the 12 mm of sterile glass coverslips in the 24 well plates.

3.3 Pour 300 µL of PDL solution (0.1 mg/mL in deionized water) in each well so that it fully covers the surface of the coverslips.

3.4 Wrap the plates with aluminum foil to prevent drying and keep it in the CO_2 incubator overnight.

3.5 The next day (before plating), aspirate the PDL solution and wash properly with 300 µL of sterile deionized water two to three times.

3.6 Add 200 µL of freshly prepared plating medium and return the plates to the incubator until plating.

4. Removal and decapitation of the fetus

NOTE: Sterilize all surgical instruments packed in aluminum foil in an autoclave at 121 °C (15 psi) for 30 min. This includes a pair of blunt-end scissors, forceps, fine forceps, two fine scissors, and one artery forceps for the entire procedure.

4.1 For generating neurons and neurospheres, use a timed-pregnant Sprague Dawley rat and mark the day with vaginal plug detection as E0.

NOTE: The culture must be performed between E14–E16.

4.2 On the day of culture, place a sterile glass Petri plate on the ice and fill it with cold Hank's Balanced Salt Solution (HBSS).

4.3 Anesthetize an E14–E16 pregnant rat with an intraperitoneal (i.p.) injection of 90 mg ketamine/kg of body weight and 10 mg xylazine/kg, then sacrifice by performing cervical dislocation.

NOTE: Rats can also be euthanized by an overdose of pentobarbital or overdose of ketamine with xylazine or diazepam.

4.4 Sterilize the dam's abdomen by spraying 70% ethanol and make a V-shaped cut in the abdominal area using sterile forceps and a pair of blunt-end scissors.

4.5 Take the embryonic sacs carefully on the Petri plate with cold HBSS solution.

NOTE: Do not use the same forceps and scissors that were just used for the skin, as this will contaminate the internal organs. Use a different set of scissors/forceps for the internal organs.

4.6 Take the embryos out of the embryonic sacs in fresh, cold HBSS.

4.7 Decapitate the head with sterile scissors.

5. Removal of brain and dissection of the cortex with hippocampus

5.1 Before starting, fill 90 mm sterile Petri dishes with cold, sterile HBSS.

5.2 Transfer the heads in the sterile dishes using sterile, blunt-ended dressing forceps.

5.3 Under the stereomicroscope, hold the head from the snout region with sterile, serrated forceps and remove the brain by cutting the skin and skull open.

5.4 Collect all the embryo brains in the same manner in the HBSS solution.

5.5 Remove all meninges from the hemispheres and midbrain by holding the brainstem.

5.6 Carefully remove the intact hemispheres resembling mushroom caps that contain the hippocampus and cortex.

5.7 Collect the hemispheres containing cortex and intact hippocampus in a 15 mL conical tube containing 10 mL of dissociation medium.

6. Dissociation of cortical and hippocampal tissue into single neurons

6.1 Allow the collected tissues to settle down and aspirate the dissociation medium, leaving 5%–10% of medium in it.

6.2 Add 10 mL of fresh dissociation medium to the tissue, and repeat step 6.1 twice.

6.3 Add 4.5 mL of dissociation medium and 0.5 mL of 0.25% (1x) trypsin EDTA (ethylene diamine tetraacetate) solution.

6.4 Keep the tissue in the incubator at 37 °C for 20 min for the digestion to proceed.

6.5 Aspirate the medium and add 10 mL of dissociation and plating medium consecutively to the digested tissues.

6.6 Allow the digested tissues to settle down and aspirate the dissociation medium. Add 2.5 mL of plating medium and pour into the base of a 90 mm sterile dish.

6.7 Triturate the digested tissues in the corner base of the dish using a 1,000 µL pipette tip to occupy the minimal volume.

6.8 Pass the obtained cell suspension through the 70 µm cell strainer, excluding any chunks of tissue.

6.9 Determine the density of viable cells using the trypan blue dye exclusion method and count the number of cells in an automated cell counter.

6.9.1. For trypan blue dye exclusion method, take 10 µL of the cell suspension and 10 µL of 0.4% trypan blue stain, mix thoroughly, and add 10 µL of the mixture in one of the two enclosed chambers of the disposable chamber slides.

6.9.2. Insert the slide containing the mixture into the cell counter and obtain the reading.

NOTE: The trypan blue dye exclusion method is based on the principle that live cells (due to their intact membranes) will exclude trypan blue dye and will hence show a clear cytoplasm, compared to a non-viable cell that will easily take up trypan blue and appear blue in color[15].

6.10 Dilute the number of cells obtained to plate 1.5 x 10^5 cells/mL for high density and 20,000 cells/mL for low density in two separate tubes containing 30 mL each of the plating medium.

6.11 Aspirate the previously added plating medium from each well and plate 500 µL of cells dispersed in plating medium in each well.

6.12 After that return the plates to the incubator at 37 °C and 5% CO_2 for 4 h.

6.13 Examine the cells for adherence under the microscope 4 h after plating.

6.14 If there is proper adherence of the cells in both plates, replace the medium in each well with 500 µL of fresh maintenance medium and incubate at 37 °C.

6.15 Culture these neurons grown at low density for 30 days by changing the maintenance medium 2x per week.

6.16 Culture the neurospheres obtained from the high-density plated neurons in the same maintenance medium by transferring them to the ultra-low attachment plates.

6.17 Characterize the neurons and the neurospheres by immunostaining them with important markers. For immunocytochemistry, first fix the cells/neurospheres using 4% formaldehyde for 30 min on the plate itself, then permeabilize the cells with 0.1% non-ionic detergent for 10 min.

6.18 Add primary antibodies for both neurons (anti-Tuj1, GFAP, O4, tau) and neurospheres (anti-Nestin, GFAP, Tuj1) in phosphate-buffered saline (PBS) at 1:300 concentrations and incubate overnight at 4 °C[16].

NOTE: The Tuj1 (class III β-tubulin) and tau are positive markers for the primary neurons, while GFAP (glial fibrillary acidic protein) and O4 (oligodendrocyte marker) are negative markers for primary neurons[17,18]. In the case of neurospheres, Tuj1, GFAP, and Nestin LL serve as positive markers[19,20].

6.19 The next day, wash the cells with PBS once or twice and add appropriate secondary antibodies in PBS AT 1:600 concentrations at RT for 2 h.

NOTE: The anti-Mouse or anti-Rabbit secondary antibodies are selected depending on the host species of the primary antibody added. It should be kept in mind that the secondary antibodies must be conjugated to fluorescence derivatives suitable for fluorescence microscopy purposes.

6.20 Wash the cells again with PBS once or twice.

6.21.1 Perform nuclear staining of the cells with Hoechst 33258 (1 mg/mL stock solution in deionized water). Prepare 0.1% Hoechst solution in PBS from the stock solution and add it to the cells.

6.21.2 Incubate the cells with 0.1% Hoechst solution for 30 min, then wash again with PBS.

6.22 Add 20 µL PBS (or mounting medium) on the slide and slowly mount the coverslip containing the stained cells over the area of the slide containing PBS. Seal the margins of the coverslip with dibutylphthalate polystyrene xylene (DPX).

6.23 Perform imaging of the fixed cells under a microscope at 10x and 40x magnification.

REPRESENTATIVE RESULTS:

In this protocol, a simple strategy has been elucidated in which variable cell plating densities from two different neural screening platforms are obtained. **Figure 1A,B** illustrates the adherence of cells after 4 h of plating the neurons in high and low density plated cells, respectively. On observing the proper adherence of the neurons as shown in **Figure 1**, the plating medium was replaced by maintenance medium in each of the wells and, thereby, returned to the incubator at 37 °C. Comparatively more cell adherence was observed in the high-density plated neurons. After 24 h of plating, both high and low density plated neurons showed elaborate neuronal extensions and synaptic interconnections, as observed in the

differential interference contrast (DIC) images in **Figure 2A,B**.

In **Figure 3A**, a phase-contrast image of the low-density plated neurons after 7 days in culture is represented. Here, the neurons have developed an elaborate synaptic network consisting of dendritic branches. These neurons can be further maintained for up to 30 days by changing the maintenance medium every 3 days with the development of more intricate neuronal networks. In **Figure 3B,C**, immunocytochemical staining was performed to reveal the neuronal nature of low density culture neurons by staining with neuronal markers Tuj1 (a marker of differentiated neurons)[21] and Tau (a marker of axons)[22], respectively. The red color in **Figure 3B** indicates the presence of Tuj1 staining, and green in **Figure 3C** represents staining in the axons of primary neurons. Purity of the neuronal culture is shown by the absence of staining of non-neuronal markers for GFAP of astrocytes (**Figure 3D**) and O4 of oligodendrocytes (**Figure 3E**). The nuclei shown in blue were stained with Hoechst 33258.

The high density plated neurons after 7 days are marked by the formation of spontaneous neurospheres, as observed in **Figure 4A,B,C,D**. After 8–10 days, distinct bridges consisting of radial glial like extensions were observed between neurospheres, as seen in **Figure 4E**. The neurospheres were richly endowed with NPCs, which coexpress markers Nestin and Tuj1[23]. The neurospeheres show positive staining of Nestin and Tuj, as shown in **Figure 5**[24]. The nuclei shown in blue was stained with Hoechst 33258. These neurospheres were able to be maintained for several weeks by culturing in ultra-low attachment plates. In **Figure 6**, the longevity of neurons cultured for about 30 days was assessed, and cell viability was measured at an interval of ~5 days using the conventional MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, in which it was found that the neurons showed more than 90% viability even after 30 days of culture.

Next, the percentage of astrocytes in the high and low density seeded cultures was assessed. Since this methodology is aimed primarily at culturing neurons, it was important to assess whether this method supports preferential growth of neurons over non-neuronal cells, especially astrocytes. The presence of a population of astrocytes was observed in the neurosphere-forming high density seeded culture, marked by the green color of GFAP staining in **Figure 7A**; though, significantly less was observed compared to the Tuj1 (red)-stained neuronal population. This was also reaffirmed by the quantitative data in **Figure 7B,** in which ~17% populations of cells were GFAP-expressing, compared to 83% of the population in Tuj1 expressing cells.

The astrocytic population was also investigated through GFAP staining, compared to neuronal population (Tuj1 staining) in low density seeded cells, for 7 continuous days. Though a significant difference in total cell number was not observed during the course of 7 days, due to low seeding, the astrocyte population was also observed to be very low (almost no or very low GFAP staining), with a majority being the neuronal population (very high Tuj1 expression) as observed in **Figure 8A**.

As shown in **Figure 8B**, quantitative analysis was performed by counting the population of

astrocytes and neurons obtained through microscopy with the help of the cellSens software, in which only ~2%–3% of the astrocyte population was initially observed. Due to the lack of suitable media and nutrients to support its growth, this population of astrocytes also slowly perished over time, whereas in the presence of optimal factors and media, the neurons rapidly took over the entire culture.

As shown in **Figure 9**, it was observed that due to the presence of NPCs, the neurospheres also expressed high amounts of astrocytes, marked by the strong green signal of GFAP staining along with a stronger Tuj1 signal. Finally, to observe whether these neurospheres expanded over time, after 1 week of high-density culture, at which point the small neurospheres started to form, a few were transferred in ultra-low attachment plates and their growth was monitored every 5 days for up to 15 days.

A live/dead cell assay was also performed using calcein AM (green) and propidium iodide (red) to check the health of the cells. It was observed that the expanding neurospheres showed a large amount of green fluorescence with no red staining, indicating no death occurring in the neurospheres for at least up to 15 days in culture, as presented in **Figure 10A**. As shown in **Figure 10B**, voluminous expansion of the neurospheres was observed at every 5 days in culture for up to 15 days. To plot the line graph representing the eventual increase in the volume of neurospheres (for each timepoint), 50 neurospheres were studied, and their averages were used to derive the neurosphere volumes at each timepoint.

FIGURE AND TABLE LEGENDS:

Figure 1: Representation of cell adherence after 4 h of plating. (**A**) Cell Adherence in High Density plated neurons. (**B**) Cell Adherence in Low Density plated neurons. Scale bar in (A,B) is 200 µm.

Figure 2: Cell morphology of neurons after 24 h of plating. (**A**) Cell morphology of the high-density plated neurons. (**B**) Cell morphology of low-density plated neurons. Scale bars in (A, B) represent 20 µm.

Figure 3: Morphology and characterization of low-density plated neurons after 7 days. (**A**) Phase-contrast image of neurons showing extensive sprouting. Scale bar represents 200 µm. Overlay images showing expression for neuronal proteins (**B**) Tuj1 (red) and (**C**) tau (green)**.** Immnunocytochemistry clearly showing absence of staining in non-neuronal proteins (**D**) GFAP (green) and (**E**) O4 (red)**.** Nuclei were stained with Hoechst 33258 (blue). Scale bars in (B, C, D, E) represent 20 µm.

Figure 4: Formation of neurospheres in high density plated neurons after 7 days. (**A**–**D**) Spontaneously generated neurospheres after 7 days in culture from the high-density plated neurons. (**E**) Formation of radial glial-like extensions between two newly formed neurospheres as indicated by black arrows. Scale bars in (A, B, C, D, E) represent 200 µm.

Figure 5: Characterization of the obtained neurospheres. Overlay image of the neurospheres showing expression for neuronal protein Tuj1 (*red*) and neural stem cell marker Nestin (green), indicating a NPC-rich population. Nuclei were stained with Hoechst 33258 (blue)**.** Scale bar represents 20 µm.

Figure 6: Cell viability of primary neurons. The bar graph represents cell viability of the primary neurons, assessed using an MTT assay for up to 30 days at 5 days intervals. Error bar represents SD of the value (*$p < 0.05$).

Figure 7: Characterization of neurosphere-forming high density cultures with neuronal marker Tuj1 and astrocyte marker GFAP. (**A**) The image shows high density seeded cells (in DIC mode), which generates neuropsheres expressing both GFAP (for astrocytes) and Tuj1 (for neurons). Nuclei were stained with Hoechst 33258. Scale bar represents 20 µm. (**B**) Bar graph represents the percentage of the population of Tuj1-expressing cells and GFAP-expressing cells in the neurosphere generating high density cells. Error bar represents SD (*$p < 0.05$).

Figure 8: Characterization of low-density plated cells for primary neuron culture with neuronal marker Tuj1 and astrocyte marker GFAP continuously up to 7 days. (**A**) The image shows the low density seeded cells in four different channels (i.e., DIC, blue channel [indicates nuclear staining by Hoechst 33258], green channel [GFAP staining], and red channel [for Tuj1 staining]) for 7 days continuously. Scale bar represents 20 µm. (**B**) The bar graph represents the percentage ratio of populations of Tuj1-expressing cells to that of GFAP-expressing cells in the low density seeded cells for primary neuron culture for 7 days. Error bar represents SD (*$p < 0.05$).

Figure 9: Immunostaining of obtained neurospheres with GFAP and Tuj1. Images of the obtained neurospheres are (**A**) in DIC mode, (**B**) nucleus staining using Hoechst 33258, (**C**) astrocyte marker GFAP (green), and (**D**) neuronal marker Tuj1 (red). Scale bar represents 20 µm.

Figure 10: Growth and live/dead cell assay of neurospheres over 15 days. (**A**) Image shows the growth of a neurosphere over 15 days at 5-day intervals in DIC mode, as well as its staining with calcein AM (green indicates live cells) and PI (propidium iodide with a red color, indicatomg dead cells). Scale bar represents 20 µm. (**B**) Graph represents the increase in size of neurospheres grown in low adherence plates over a period of 15 days at 5 day intervals. Error bar represents SD.

DISCUSSION:

This protocol describes the altering of cell plating densities of primary neurons using two variable neuronal platforms that are obtained. Though this is a simple method, each step must be meticulously performed to achieve the desired results. Other previous methods have either reported long-term primary neuron cultures or neurosphere cultures. Most primary neuron culture protocols have involved the culturing of hippocampal neurons for 3–5 weeks, but most

have failed, as the neurons die and wither away due to loss of connections. Another advantage of the protocol is that the neurons can be cultured without the need for any glial feeder layer, hence maintaining purity of the neurons.

However, several critical steps must be carefully followed to obtain the desired results. First, maintaining sterile conditions throughout is absolutely necessary. It is advised to perform most steps in a laminar hood as well as pre-clean all slabs, instruments, and surgical tools with 70% alcohol before starting; otherwise, there is a higher chance of failure due to contamination by bacteria and fungi. Next, it is important to isolate E14–16 embryos; hence, the vaginal plug detection step should be carefully performed. As the embryonic day increases, the higher the chances are of contamination by non-neuronal cells. Complete removal of meninges from the hemispheres is extremely critical to reduce interference in culture by non-neuronal cells. Both plating and maintenance medium must be freshly prepared with all the components, as every component plays an essential role. Another factor that must be kept in mind is that the primary neurons obtained must be maintained by changing the maintenance medium 2x per week so that the nutrient supply to the proliferating neurons remains constant.

Although it has not yet been attempted, this protocol with slight modifications may also be useful in mouse embryonic neurons. If the desired neurons or neurospheres are not obtained following this technique, there are a few troubleshooting tips that may be helpful. To keep the tissues viable, the dissection must be performed in ice-cold HBSS. The dissection can also be performed in ice-cold Krebs buffer instead of HBSS buffer. Quickly performing the dissection is key to maintaining tissue viability. Usage of 10x trypsin will lead to overdigestion of the tissue. Hence, 10x trypsin-EDTA solution should be diluted to 1x in dissociation buffer prior to digestion. The addition of ice-cold medium to the cells, inducing freeze-shock, should be avoided at all costs, and medium after reaching RT should be used instead. Most importantly, the coverslips should always be coated with PDL, otherwise the neurons will not attach to the coverslips. In case of any difficulty while performing the trituration step (i.e., tissue not digesting properly), digestion can be performed by adding 0.5 mL of 1% DNase for 10 min. If high degrees of contamination by non-neuronal cells are encountered, ~5 µM cytosine arabinoside (araC) should be added to prevent the growth of non-neuronal cells.

Despite its multiple advantages, this technique suffers from a few limitations. It is known that this technique spontaneously generates neurospheres (although, the triggering molecular mechanisms are not known); however, few ambiguities regarding this technique remain, such as the exact size of the neurospheres formed and exact number of days required to form a sufficient number of neurospheres. Mostly, the size is the problem. Even though it has been observed that neurospheres expand in volume over time, the initial neurospheres obtained are of variable sizes. Though useful, it makes it difficult to perform a synchronized study. What distinguishes this neurosphere generation protocol from others is its robustness and simplicity. There are earlier reported protocols for culturing and propagation of neuropheres requiring special medium requirements and culture conditions, none of which is required in this protocol. In these previously reported protocols, there is hardly any uniformity for those looking to generate neurospheres.

Overall, this protocol describes a unique strategy for the generation of both 2D and 3D neuronal platforms by simply altering the cell plating densities of primary neurons isolated from embryos of E14–E16 Sprague Dawley rats. This method is cost-effective in comparison to other methods, as it can be performed with a simple set-up and requires much less reagents and steps. It can provide various applications of interest for neuroscientists. This can be used for screening platforms for various neuro-therapeutic leads, observing the roles of various neuronal cargo proteins, investigations of cellular pathways in many neurodegenerative diseases, and many other applications. The neurospheres can be further used for the screening of various neural differentiating agents and studying the early stages of neural development in vitro[25,26].

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DISCLOSURES:

The authors declare no competing financial interests.

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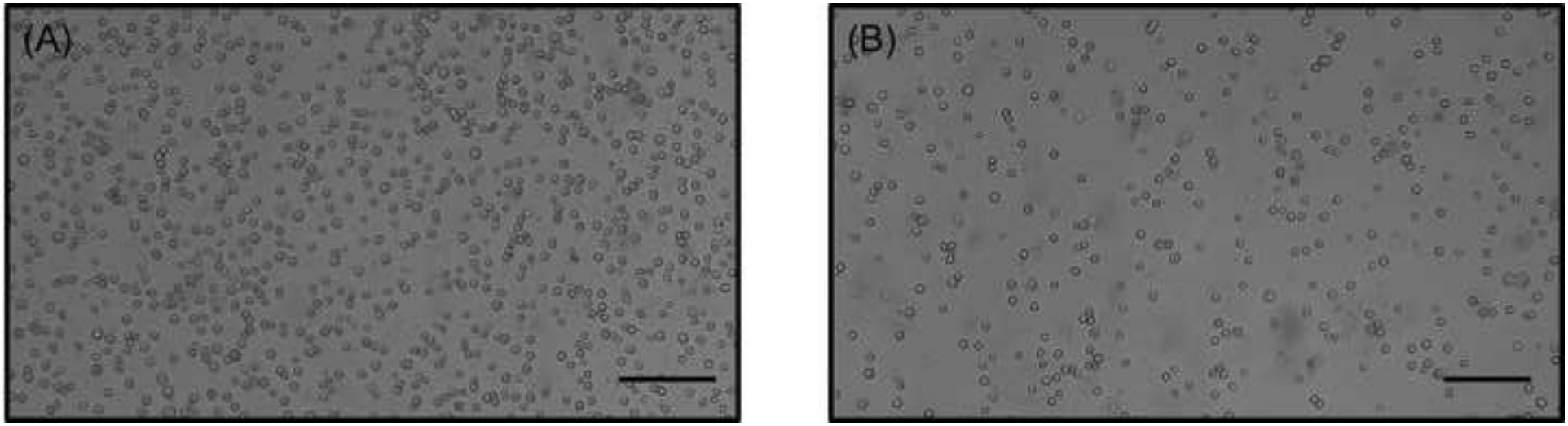

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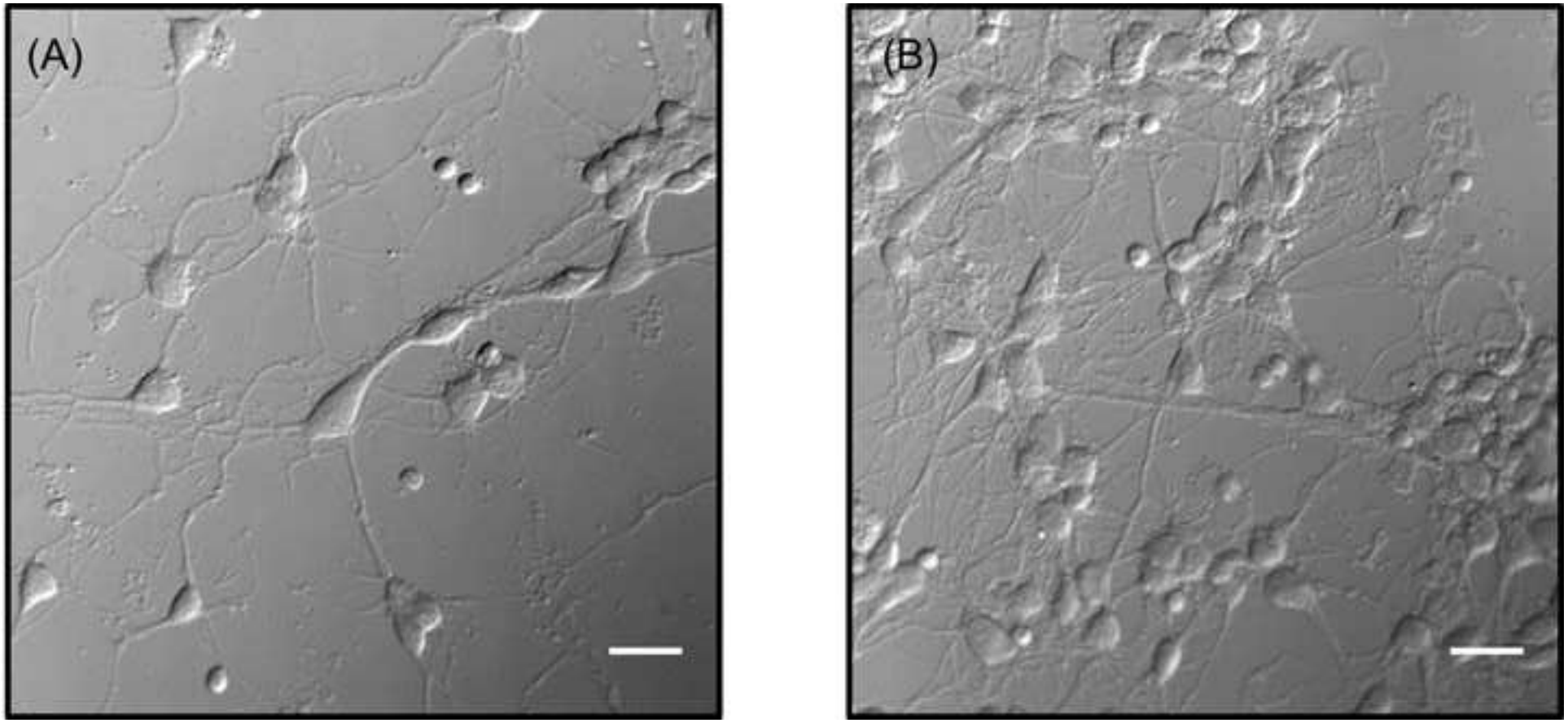

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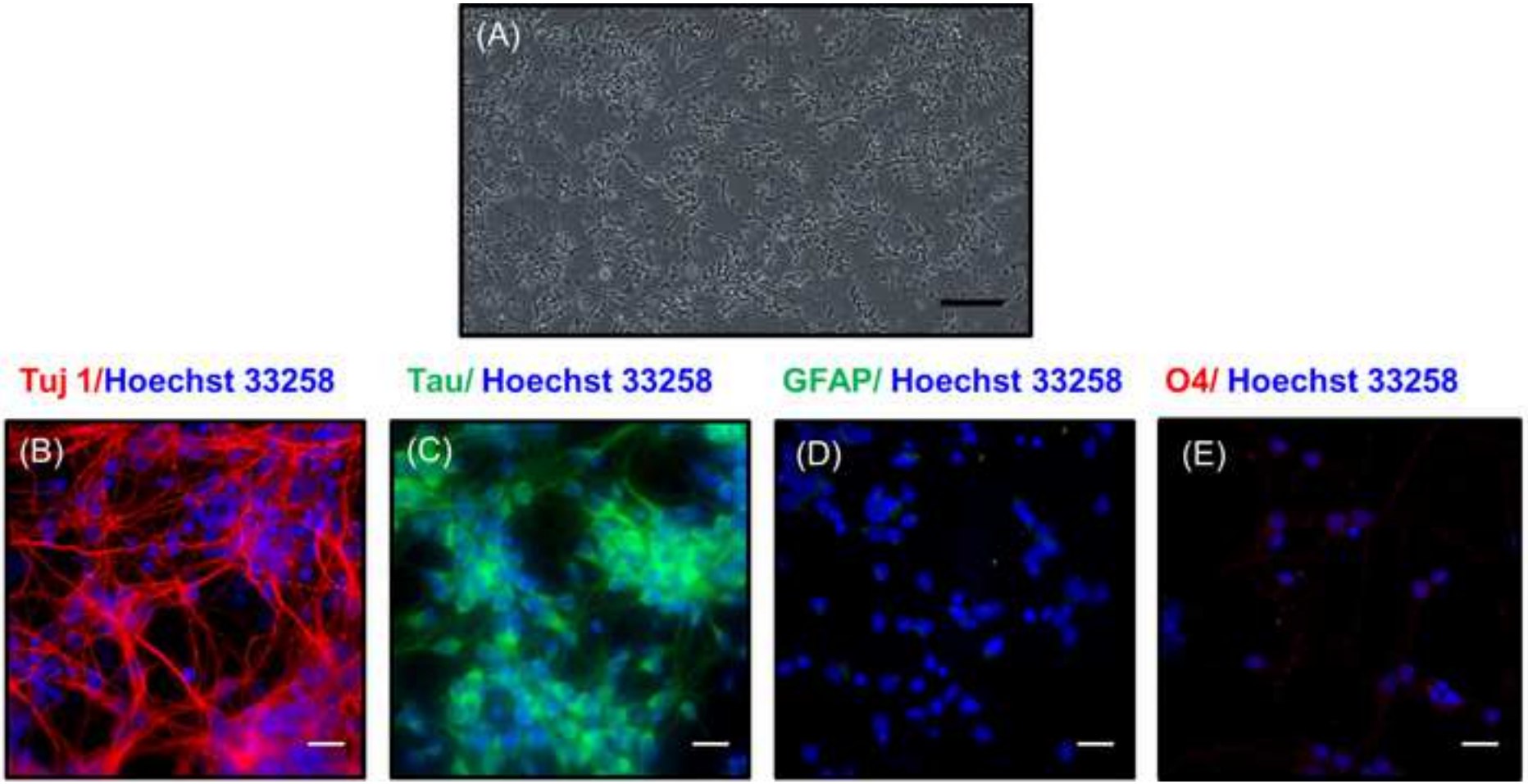

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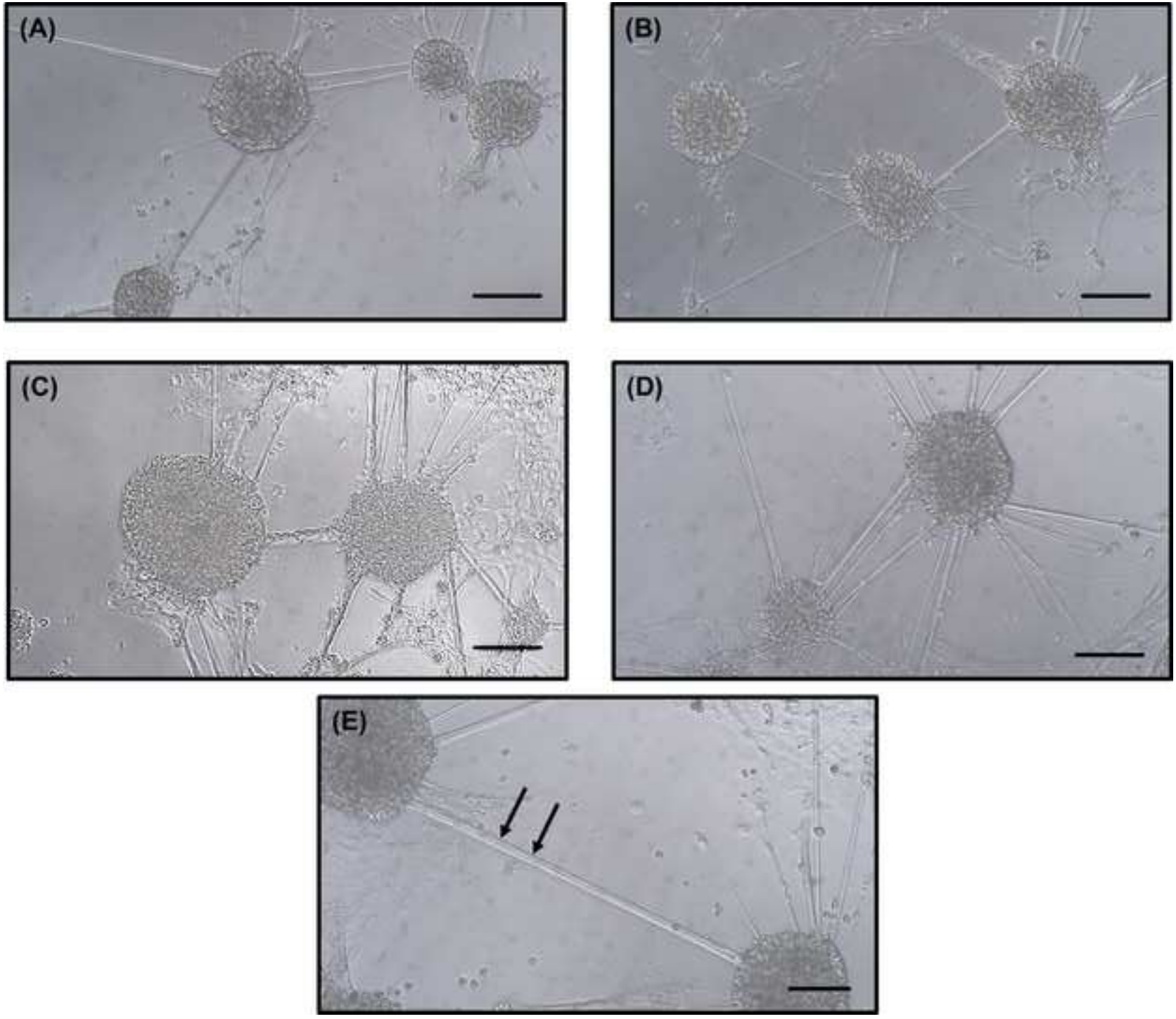

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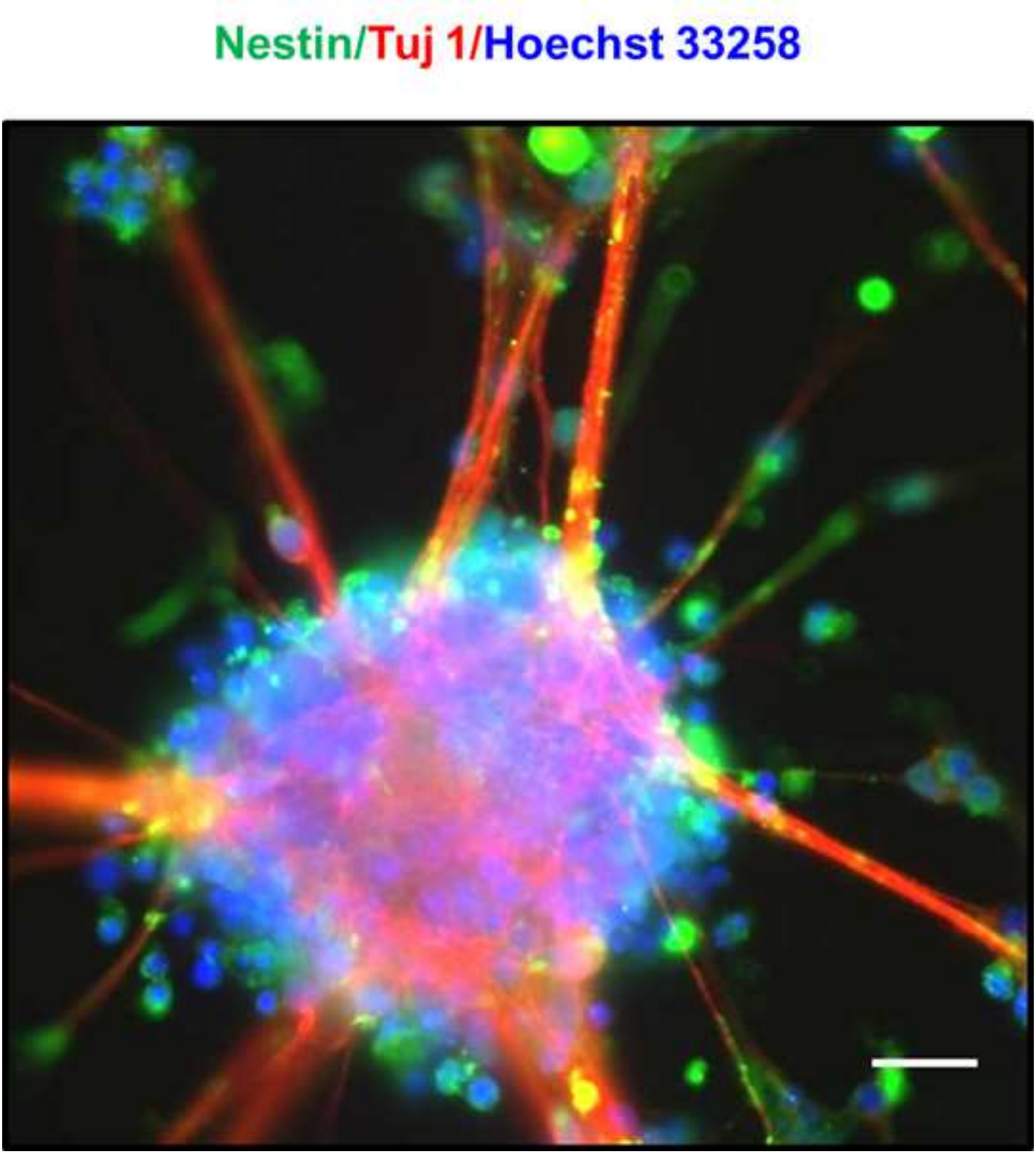

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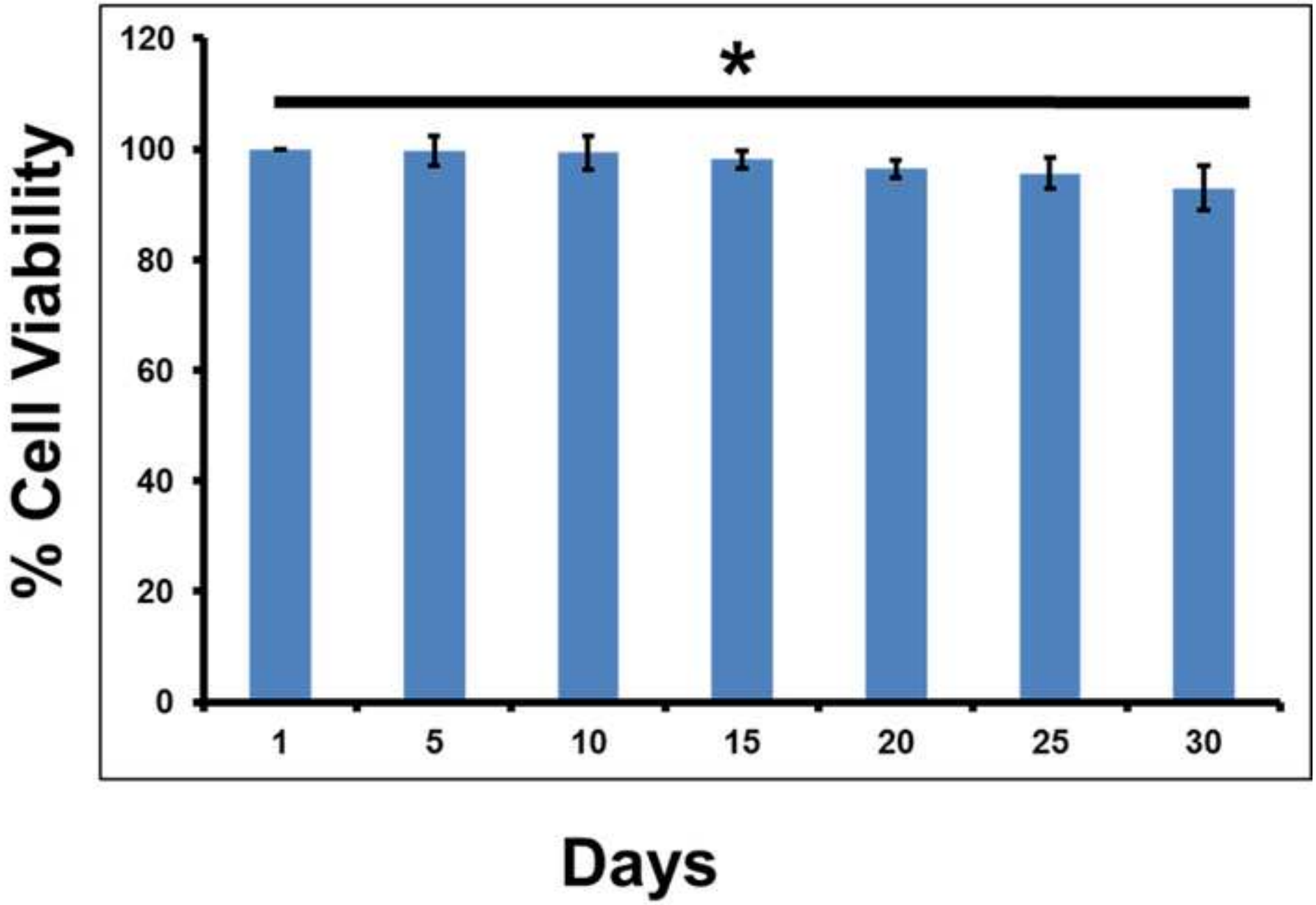

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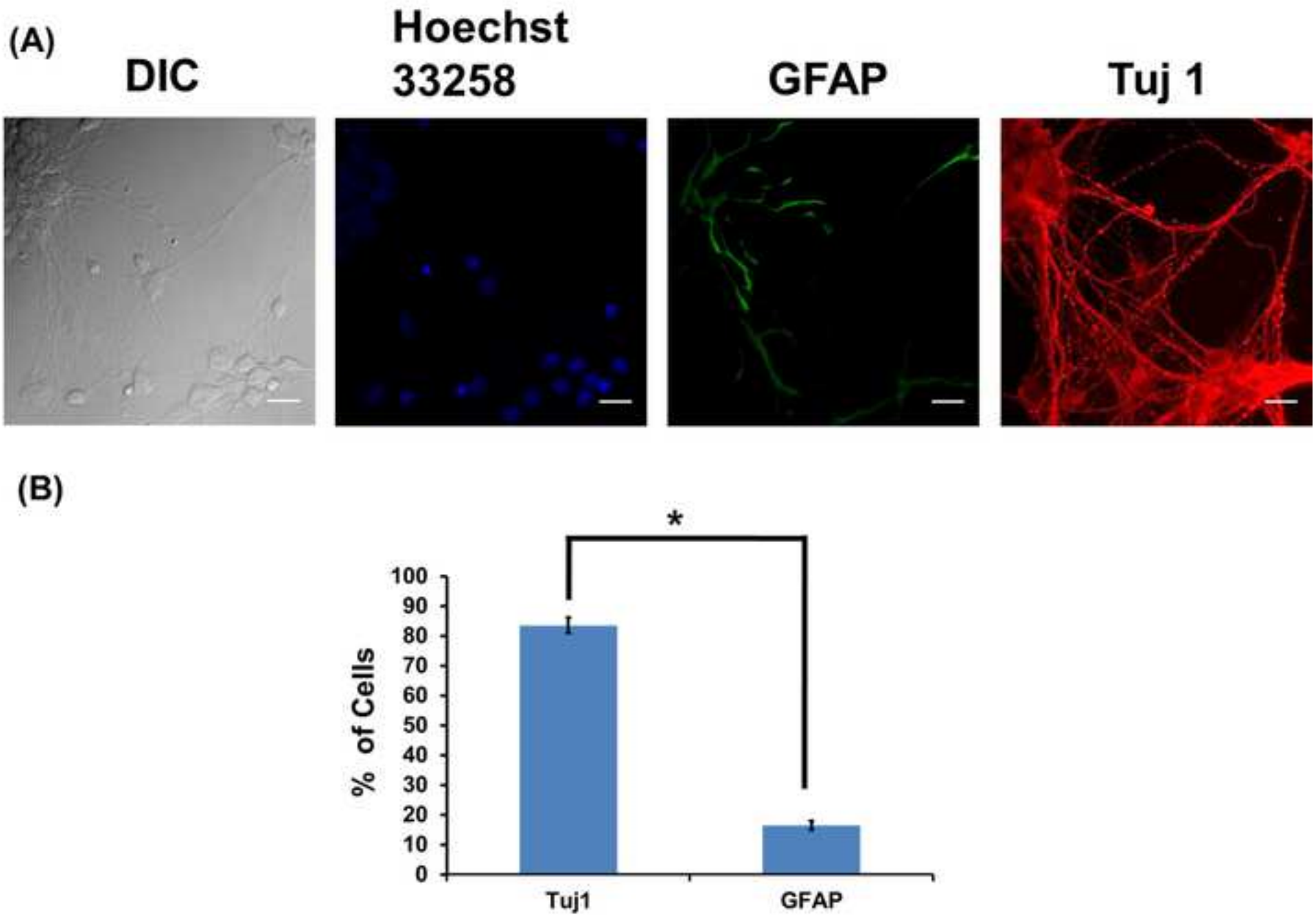

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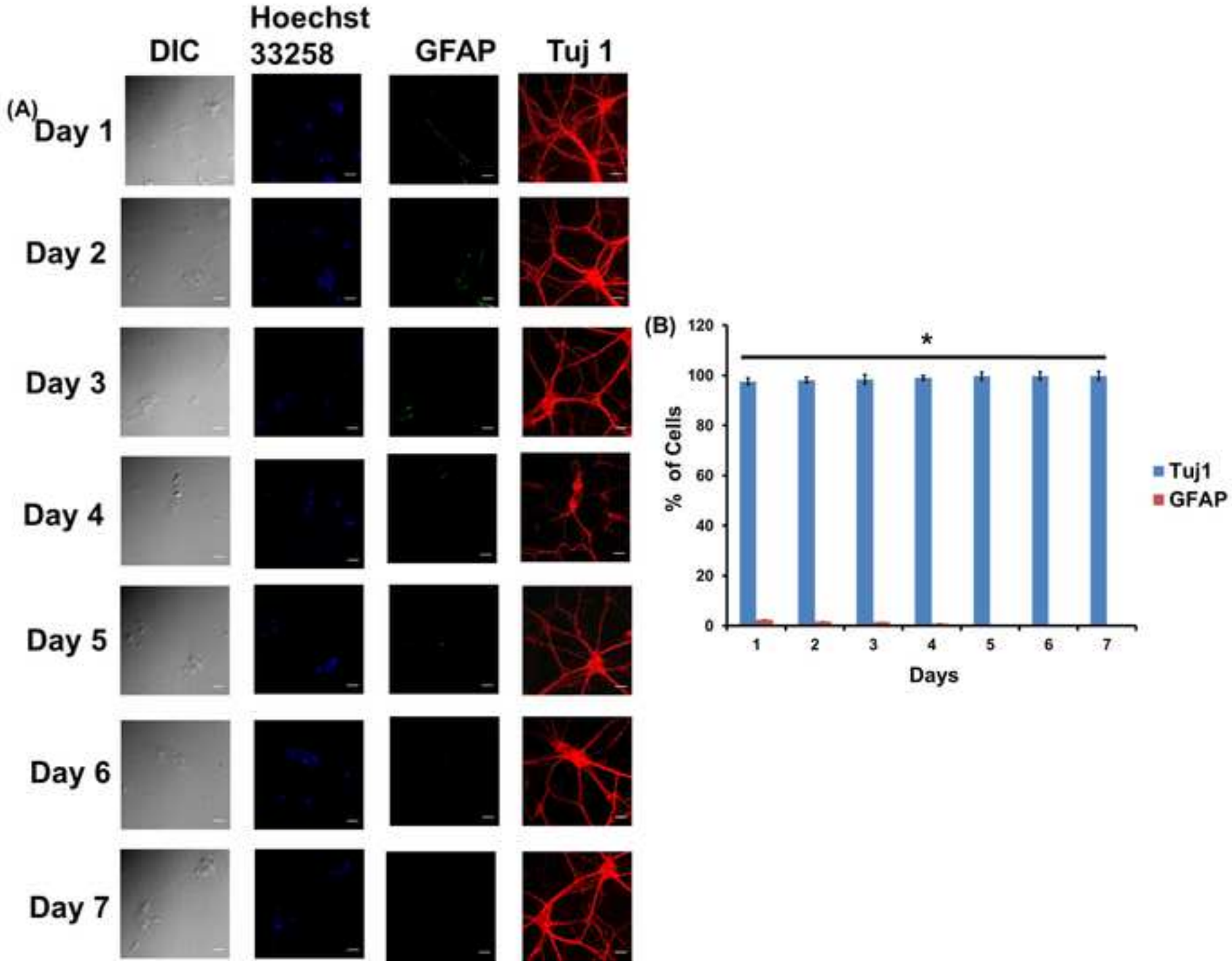

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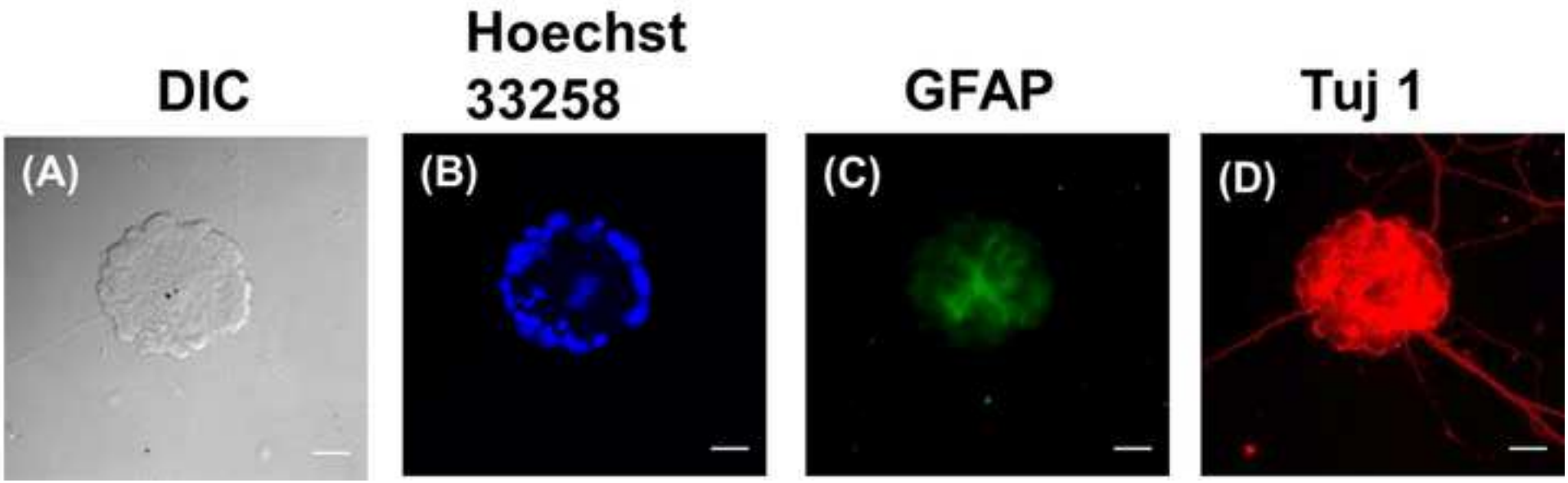

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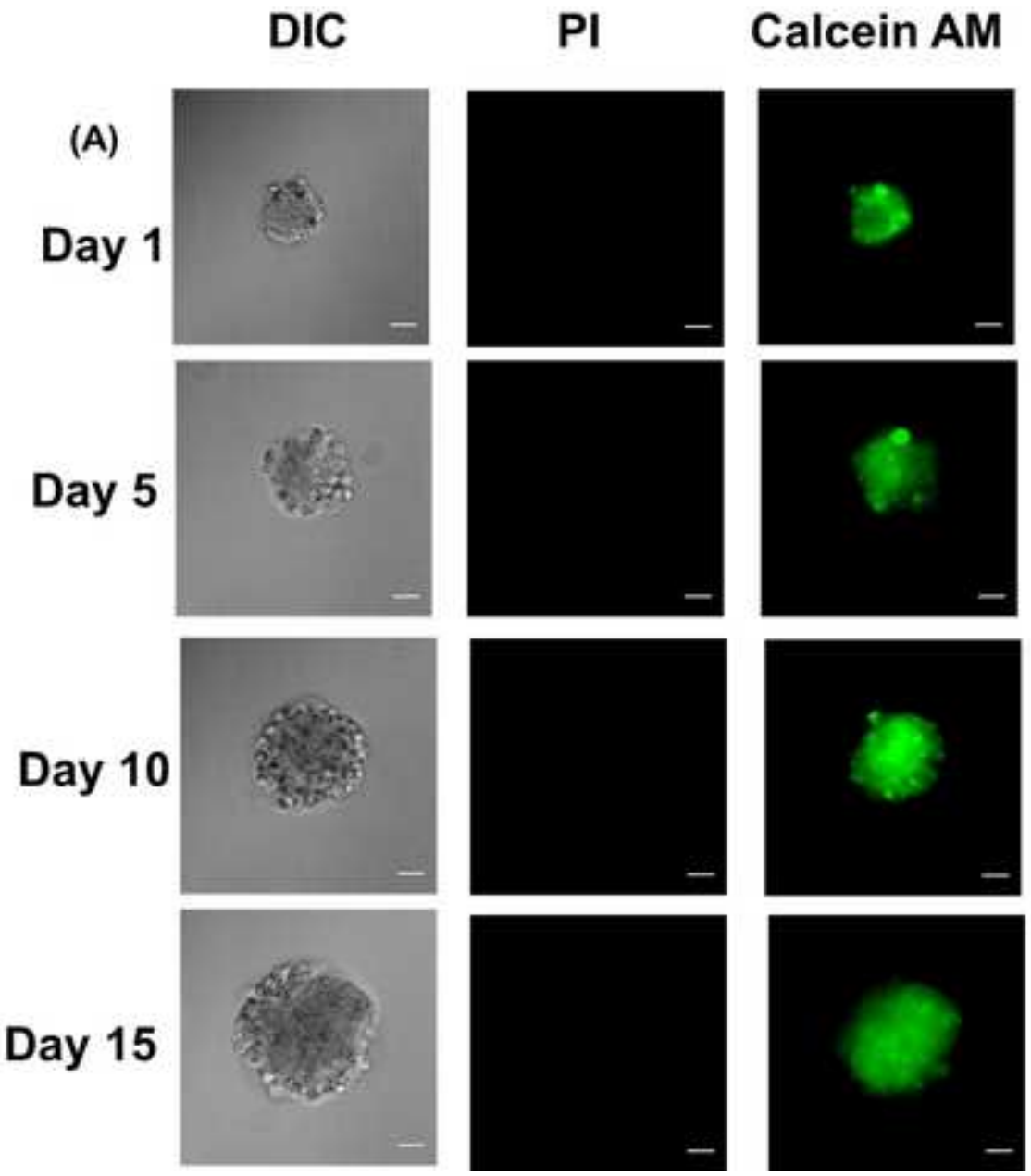

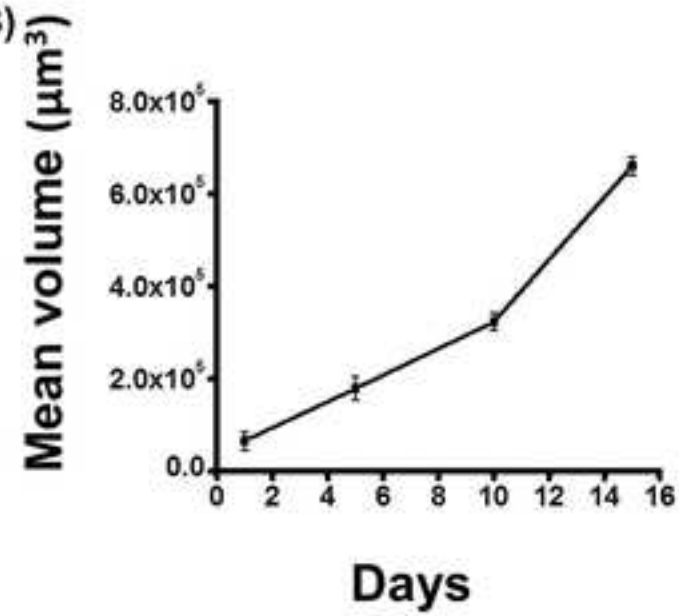

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Anti- Nestin	Abcam	AB92391
Anti-O4	Millipore	MAB345
Anti-Tau	Abcam	AB76128
Anti-Tuj1	Millipore	MAB1637
B27 Serum Free Supplement	Gibco	17504-044
Cell Counter	Life technologies	Countess II FL
CO2 Incubator	Eppendorf	Galaxy 170 R
D-glucose	SDFCL	38450-K05
Ethanol	Merck Millipore	100983
Fluorescence Microscope	Olympus	IX83 Model
Formaldehyde	Sigma Aldrich	47608
GlutaMax-I Supplement	Gibco	35050-061
GtXMs IgG Fluor	Millipore	AP1814
GtXMs IgG (H+L)	Millipore	AP124C
HEPES	SRL	16826
Hoechst 33258	Calbiochem	382061
Horse Serum	HiMedia	RM10674
Hydrochloric Acid	Rankem	H0100
Laminar Hood	BioBase	BBS-V1800
MEM Eagle's with Earle's BSS	Sigma Aldrich	M-2279
Microscope	Dewinter	Victory Model
Neurobasal Medium	Gibco	21103-049
Plasticware (24 well plate, cell strainers, and low adherence plates)	BD Falcon	353047, 352350 and 3471
90 mm Petridishes	Himedia	PW001
Penicillin/Streptomycin	Gibco	15140-122
Poly-D-Lysine	Millipore	A.003.E
Potassium Chloride	Fisher Scientific	BP366-500
Potassium Phosphate Monobasic	Merck	MI6M562401
Sodium Chloride	Qualigem	15918
Sodium Phosphate Dibasic	Merck	MI6M562328
Stereomicrosope	Dewinter	Zoomstar Model
Triton-X 100	SRL	2020130
Trypan Blue Solution	Gibco	15250-061
0.25 % Trypsin-EDTA	Gibco	25200-072

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   Reply: We have made this change in the video now.

3. 6:13: Please reformat to ~2.51 x 107 cells/mL

Reply: We have reformatted it in the new video.

4. 7:05, 8:24: Please leave a single space between 200 µm and 20 µm.

   Reply: We have made this correction and it is now reflected in the new video.

5. Please include all the result figures both in the text and the video in the representative result section. For example, few figures are not shown in the text (8:57) and figures 6-10 are not shown in the video.

   Reply: We have included all the figures from (6-10) in the video now along with narration and the figure (8:57) has been included in the text as figure 4. It can be tracked in the highlighted manuscript marked red.

6. Once done, please ensure that the video is no more than 15 min and contains 59800R2 in the name.
   Reply: The new video is less than 13 min and contains 59800R2 in the name.
7. Production comment: There is still one jump cut 4:12 that should be replaced with a crossfade.

   Reply: The jump cut at 4:12 has been replaced with crossfade. .