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14. ABSTRACT: Hearing loss is a major clinical complaint of military personnel who are exposed to loud noise known to damage cochlear hair cells and auditory neurons. Our long-term goal is to mitigate this damage through "next generation" cochlear implants (CIs) that incorporate transplanted human stem cells guided to become new, functional auditory neurons. By combining clinically proven CI methodologies with developing stem-cell based neural replacement strategies, we seek to achieve large gains in CI performance by establishing a new intracochlear neural network to improve CI-neuron connectivity, using the CI to stimulate this network. Our work advances translational efforts by (1) improving intracochlear transplantation of stem-cell derived auditory neurons through innovative three-dimensional multicellular aggregates and (2) promoting survival, neural plasticity, and synaptogenesis through supportive neurotrophins and extracellular matrices. We expect that transplanted stem-cell-derived neurons will establish new synaptic connections with surviving auditory neurons. We will focus on *in vitro* experiment using human pluripotent stem cell lines in the first year. We will then move on to *in vivo* animal experiments in the second and third year to evaluate our research aims.

15. SUBJECT TERMS
hearing restoration, stem cell regeneration, regenerative engineering, regenerative medicine, material science

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Unclassified	Unclassified	Unclassified	Unclassified	40	19b. TELEPHONE NUMBER *(include area code)* 312-503-7955

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

The Veterans Administration reports that hearing loss is among the top medical concerns of service personnel. Our proposed work addresses key roadblocks in the way of translating our "Biohybrid Cochlear Implant (CI)" design to new users. We will evaluate three solutions for improving stem-cell survival and synaptogenesis to the surviving spiral ganglion neuron population, which will benefit all stem-cell-based remedies, regardless of implementing the biohybrid CI concept.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

hearing restoration, stem cell regeneration, regenerative engineering, regenerative medicine, material science, spiral ganglion neurons, cochlear implant, biohybrid implant, hydrogel

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1: Optimize the delivery of hPSC-derived multicellular aggregates using a simple *in vitro* model

Major Task 1: Optimize delivery of hPSC-derived 3-D multicellular aggregates using a simple In vitro model.

Subtask 1: Achieving Northwestern University IACUC and ACURO approvals (Month 1-3).
Status: completed as of 12/31/2018.

Subtask 2: 3-D multicellular aggregates preparation (generation of inner ear organoids) Human embryonic cell lines: WA25 (WiCell, WI) (Month 1-2).
Status: in progress (90 % completed as of 10/31/2019 at Indiana University school of Medicine).

Subtask 3: Three-dimensional multicellular aggregates preparation (generation of auditory neuron spheroids). Human embryonic cell lines: H1, H7, and H9 (WiCell, WI) (Month 1-3).
Status: completed as of 12/31/2018.

Subtask 4 and 6: Evaluate transfer of spheroids/organoids with a volume flow system using Human embryonic cell lines: H1 H7, H9, and WA25 (WiCell, WI) (Month 1-3).
Status: completed as of 12/31/2018.

Subtask 5 and 7: Evaluate transfer of hESC-derived ONP spheroids/organoids using the pressure regulation system using human embryonic cell lines: H1, H7, H9, and WA25 (WiCell, WI) (Month 1-3).

Status: completed as of 10/31/2019.

Subtask 8: Outcome analysis (ANOVA) Possible additional measurements in selective experimental conditions, and writing up results (Month 3-4).
Status: in progress (90% completed as of /10/31/2019).

Specific Aim 2: Create supportive biochemical stem cell niches for successful trans-plantation of hPSC-derived 3-D multicellular aggregates into the cochlea.

Major Task 2: Optimize delivery of hPSC-derived 3-D multicellular aggregates using a simple In vitro model.

Subtask 9: 3-D multicellular aggregates preparation (generation of inner-ear organoids) Human embryonic cell lines: WA25 (WiCell, WI) (Month 3-12).
Status: in progress (90% completed as of 10/31/2019).

Subtask 10: 3-D multicellular aggregates preparation (generation of auditory neuron spheroids). Human embryonic cell lines: H1, H7, and H9 (WiCell, WI) (Month 3-12)
Status. completed as of 10/31/2019.

Major Task 3: *In vivo* development of supportive stem-cell niches.

Subtask 11: Optimize the number of PODS in nanofibrillar cellulose (NFC) hydrogels for 3-D multicellular aggregates (Month 2-4).
Status: in progress (75% completed as of 10/31/2019).

Subtask 12: 3-D multicellular aggregates preparation (generation of inner-ear organoids) Human embryonic cell lines: WA25 (WiCell, WI) (Month 13-36)
Status: not started yet as of 10/31/2019.

Subtask 13: 3-D multicellular aggregates preparation (generation of auditory neuron spheroids). Human embryonic cell lines: H1, H7, and H9 (WiCell, WI).
Status: in progress. (50% completed as of 10/31/2019).

Subtask 14: Transplantation of spheroids in the DTR^+ mice inner ear using the volume-controlled system.
Status: completed as of 10/31/2019.

Subtask 15: Transplantation of organoids in the DTR^+ mice inner ear using the pressure-controlled system
Status: not started yet as of 10/31/2019.

Subtask 16: Transplantation of spheroids in the DTR^+ mice inner ear using volume-controlled system
Status: not started yet as of 10/31/2019.

Subtask 17: Transplantation of organoids in the DTR^+ mice inner ear using pressure-controlled system
Status: not started yet as of 10/31/2019.

Subtask 18: Sacrifice and histological assessments of the chronically implanted DTR^+ mice (1, 2, and 3 month survival periods)

Status: in progress (20% completed as of 10/31/2019).

Subtask 19: Sacrifice and histological assessments of the chronically implanted DTR$^+$ mice (1-month survival period (n=40), 2-month survival period (n=40), and 3-month survival period (n=40)) (Month 4-33)
Status: in progress (30% completed as of 10/31/2019).

Subtask 20: Sacrifice and histological assessments of the DTR$^+$ mice without DT injection (non-deafened control (n=40)) (Month 4-33).
Status: in progress (20% completed as of 10/31/2019).

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting.

1) Major Activities
Our Major Task 1 is to optimize delivery of hPSC-derived 3-D multicellular aggregates (3-D neuronal progenitor spheroids, neurons, and organoids) using an in vitro model. We have completed analysis of the first set of experiments. Our Major Task 2 is to accomplish *in vitro* development of supportive stem-cell niches. We have started characterized hESC-derived 3-D multicellular aggregates using immunocytochemistry. We have also started optimizing the number of PODs in nanofibrillar cellulose hydrogels. Our Major task 3 is in vivo development of supportive stem-cell niches.

2) Specific Objectives
In Aim 1, we develop effective means of transplanting stem-cell derived SGNs into the scala tympani. In Aim 2, we will bioengineer and evaluate supportive niches to improve cell survival and neurite growth to improve the neural selectivity of CI electrodes. These aims are essential to the biohybrid CI, which leverages proven CI technology with the parallel transplantation of stem-cell-derived SGNs into the scala tympani.

3) Significant Result & Key outcomes
Specific Aim 1:
Preparation of human ESC-derived ONP spheroids: H7 and H9-derived late-staged ONPs (LONPs) were first seeded onto a conventional monolayer-culture plate coated with rLaminin-511 (iMatrix-511^{TM}, Nakalai USA, San Diego, CA). After 48-72 hours in culture, LONPs were single -suspended and plated into a 3-D spheroid-forming culture device at a seeding density of $2x10^6$ cells/mL. They were then either seeded into EZSPHERE^{TM} (Nakalai USA, San Diego, CA) culture microwells or a 96-well U-bottom culture plate (Fisher Scientific, Waltham, MA, USA) to produce spheroid aggregates for 72-96 hours. For our purpose, we aimed to generate LONP-spheroids; the size of which ranged between 200-800 μm in size. Human-ESC-derived ONP Spheroids were suspended in culture dishes containing ONP maintenance medium that has been previously reported. Figure 1 summarizes this protocol.

Transfer equipment: Micropipettes were formed from 1.5 mm O.D. x 0.84 mm I.D. glass and a Sutter P-97 puller (Sutter Instrument, Novato, CA) forming tips by the "score-and-break technique" [1]. Pipette tip diameters were selected for each transfer method: for the volume-flow method, I.D.s 100-150 % of the aggregate size were chosen, while a range of I.D.s were evaluated for the negative-pressure method. Pipettes were held in position with a WPI, Inc. Micropipette Injection Assembly (WPI Precision Instruments, LLC, Sarasota, FL) attached to a Narishige MHW-3 3-axis micromanipulator (Narishige USA., Amityville, NY). Culture plates were positioned on a custom-built stage. For negative-pressure holding, we used a Sutter Instrument XenoWorks digital microinjector (Sutter Instrument, Novato, CA) while for volume-flow transfer, we will use a WPI UMP3 UltraMicroPump (WPI Precision Instruments, LLC, Sarasota, FL).

Results: Assessment of hESC-derived spheroid aggregates transferred from source to destination dish by manipulating volume flow were first performed by using the aggregate size ranged from 200-800 μm in size while changing flow rate between 50 nl/sec to 200 nl/sec. Although feasible, we soon realized that controlling the exact flow rate in a scale of this magnitude (i.e., 50nl/sec to 200nl/sec) with precision was rather challenging. We speculate that this was partly due to the technical limitation of the WPI UMP3 UltraMicroPump. Despite this challenge. we were able to identify that the micropipette inner diameter needed to be set at 116% ± 5.4 (n = 30) of the diameter of spheroids to be able to transfer the spheroids from the source to a destination dish.

We then proceed to the negative pressure approach. The initial value of micropipette inner diameter was set at approximately 15% of the aggregate diameter, based on values used for *in vitro* fertilization [1,2] and our pilot study, which showed that spheroid capture is feasible with this value. Figure 2-6 show microphtographical images of the four sets of hESC-derived ONP spheroids that were transferred on 12/05/2018 (Figure 2) and 12/06/2018 (Figure 3-6). Note the shape of "Failure" pattern Figure 6.

Our initial quantification of hESC-derived spheroid transfer using the negative pressure method indicated three "failure" patterns out of 61 transfers (success rate of 95.1%). Here, Failure" pattern is defined as a "significant" morphological change after the transfer. As compared with our negative pressure method, the controlled flow method showed 33 Failure patterns out of 60 transfers (success rate of 55 %). **It became apparent that our negative pressure method is superior to the volume-flow method.**

We then went on further performing a series of quantitative analysis on our hESC-derived ONP spheroids data. Figure 6 indicates a histogram of hESC derived ONP spheroids that were used in our spheroids transfer experiments performed on Dec.05 and Dec. 06, 2019, respectively. Here, an average diameter of a spheroid is defined as follows.

$$Spheroid\ Diameter\ (um) = + \frac{Larger\ diameter\ \ (um) + Smaller\ diameter\ (um)}{2}$$

Using this equation, we have measured the diameter of all of hESC-derived ONP spheroids using Microscope Imaging Software (Leica Microsystems Inc., Buffalo Grove, IL, USA). Figure 7 shows a histogram of a spheroid diameter measured on Dec 5th, 2019 (A) and Dec

6th, 2019 (B). The batch that was used on Dec. 05, 2019 contains smaller diameter with an average diameter = 347 ± 45.5 um as compare with the one on Dec. 6th 2019 with an average diameter = 454.4 ±67.4 um. This difference might be due to the duration in culture for a spheroid formation. The spheroids on Dec 5th were in culture for 72 hours and those on Dec 6th were in 96 hours.

Figure 7 shows a multiple scatter plots on hESC-derived ONP spheroid transfer experiments. We analyzed the data that were combined two sets of data (i.e., Dec 5th, 2018 and Dec 6th, 2018). Figure 8A shows a relationship between a ratio of a pipette inner diameter (I.D.) to a spheroid diameter, and a spheroid diameter (um). It appears that larger spheroid diameter with smaller pipette I.D./spheroid diameter resulted in unsuccssrul transfer. Also, it appears that lager spheroid diameter in general resulted in a unsuccessful tranfer. In other words, a spheroid diameter between 280 um and 360 um resulted in a successful transfer as compared with a sheroid dismeter betweeb 380 um and 600 um. Figure 8B shows a relationsghip between a ratio of a pipette I.D. to a spheroid diameter and a micropipette I.D. (um). Micropippte diameter of 100-120 um appears to result in successful transfer. Figure 8C shows a relationship between Delta P and a ratio of pipetter I.D to spheroid diameter. Here, delta P is defined as follows.

$$Delta\ P\ (hPa) = Pickup\ pressure\ (hPa) - Releasing\ pressure\ (hPa)$$

Figure 8C indicates that delta P and ratio of pipette I.D. to spheroid diameter does not appear to be related each other. Note that delta P can be defined only when both pickup pressure and releasing pressure was determined (i.e., delta P can only be calculated in "successful" transfer). Figure 8D indicates a relationship between a pickup pressure and a micropipette diameter. This fugure demostartes that a smaller diameter of micropipette I.D. such as 77 um and 93 um had a unsuccessul tranfer whereas the diameter of micropipette I.D. above 100 um does not indicate any unsuccessful failure. Finally, Figure 8E indicates a relationship between delta P and micropipette I.D. Once again, it appears that there is weak correlation between delta P and micropipette I.D.

Figure 9 indicates the range of a micro-glass pipette pressure (hPa) that was used to transfer a hESC-derived ONP spheroids in relation to the ONP diameter (um) with 6 different pipette I.D. size (um). These data suggested that the micro-glass pipette pressure exponentially (negative) reduces in relation to the micro-glass pipette I.D. (see a hypothetical model on Figure 10).

Based on our findings stated above, we then performed the third set of hESC-derived ONPs spheroid transfer experiment using the negative pressure method on 04/11/2019. The third set of an experiment provided us with sufficient statistical power to determine the appropriate size of a glass pipette tip and adequate negative pressure for the transfer, and also the favorable diameter of the spheroids for the transfer. We have performed 55 hESC-derived ONP spheroid transfer on 04/11/2019. Figure 11 demonstrates a series of quantitative analysis on hESC-derived ONP spheroid transfer experiments on April 11th, 2019. The data once again demonstrated that as pipette inner diameter increases, less negative pressure is required for spheroid capture. Spheroid diameter histogram indicated that ONP spheroids that were used in this particular experiment ranged from 520μm to 860μm. In addition, pressure differential

analysis demonstrates that as the pipette inner diameter increases, the difference between the spheroid capture and release pressures tended to drops. Finally, the pressure differential by pipette/spheroid ratio indicated that the larger the ratio of pipette inner diameter to spheroid diameter, the lower the pressure differential required for spheroid transfer.

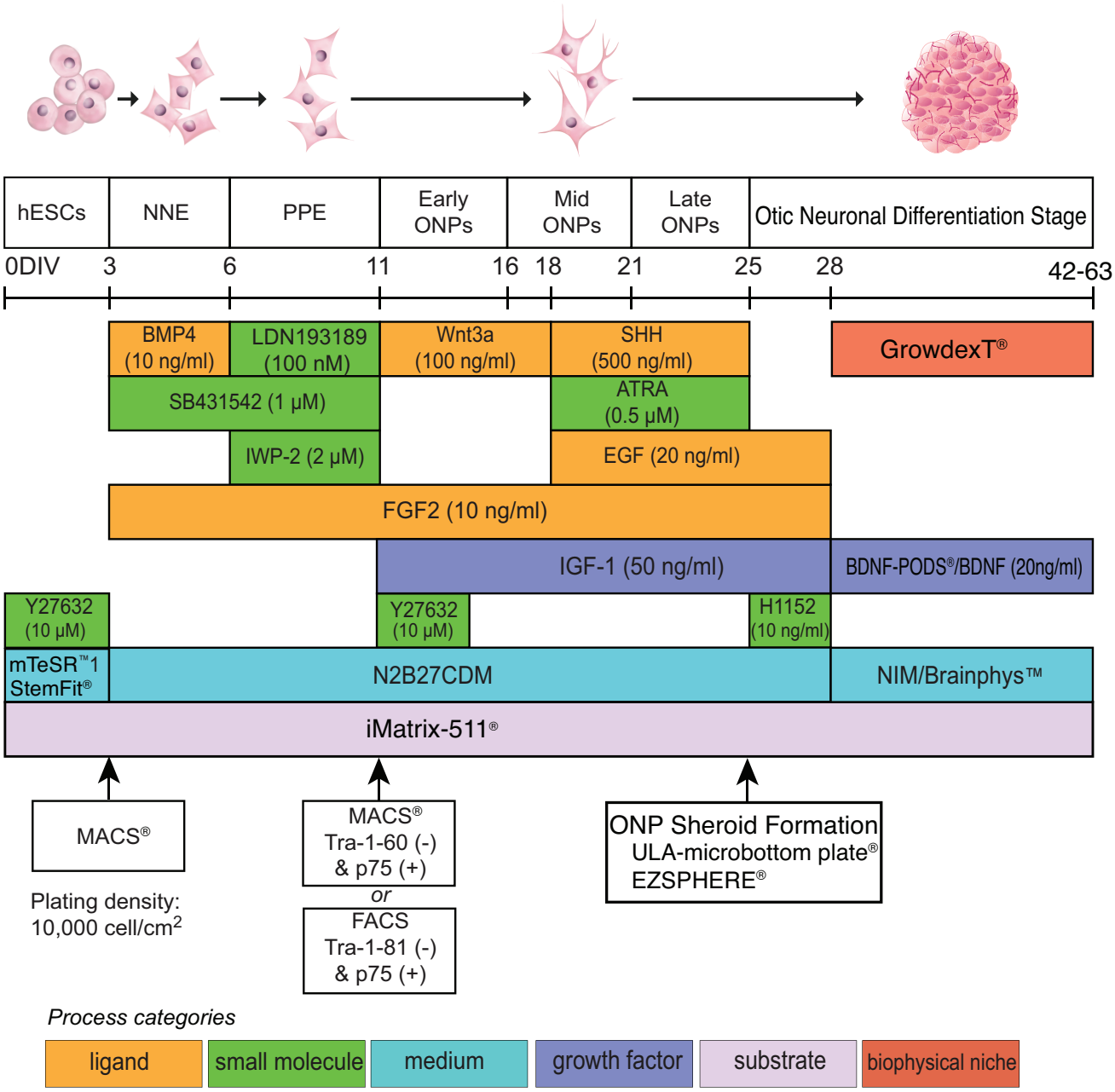


Figure 1: Schematic summary of the protocol and timeline for deriving otic-lineage neurons from undifferentiated hESCs. DIV: day in vitro; NNE: nonneuronal ectoderm; PPE: preplacodal ectoderm; ONP: otic neural progenitor; BMP4: bone morphogenetic protein 4; SHH: Sonic hedgehog; ATRA: all-trans retinoic acid; EGF: epidermal growth factor; BDNF: brain-derived neurotrophic factor; PODS®: PoLyhedrin delivery system; IGF-1: insulin- like growth factor 1; FGF2: fibroblast growth factor 2; E8: N2B27-CDM: chemically defined medium containing N2™ and B27™ supplements; StemFit®: StemFit® Basic O2; MACS®: magnetic-activated cell sorting; FACS: fluorescence-activated cell sorting; and p75: low-affinity neurotrophin receptor (p75NTR). Protocol adapted and modified from Matsuoka et al. [3,4]

Figure 2: Images of spheroids transferred on 12/05/2018. The transferred spheroid is noted by the arrow. Each interval of the scale (upper right) indicates 100 m. The scintillation observed at spheroid peripheries are caused by light reflection of individual cells. In cases of ovoid spheroids, diameter is estimated by the mean of major and minor axes.

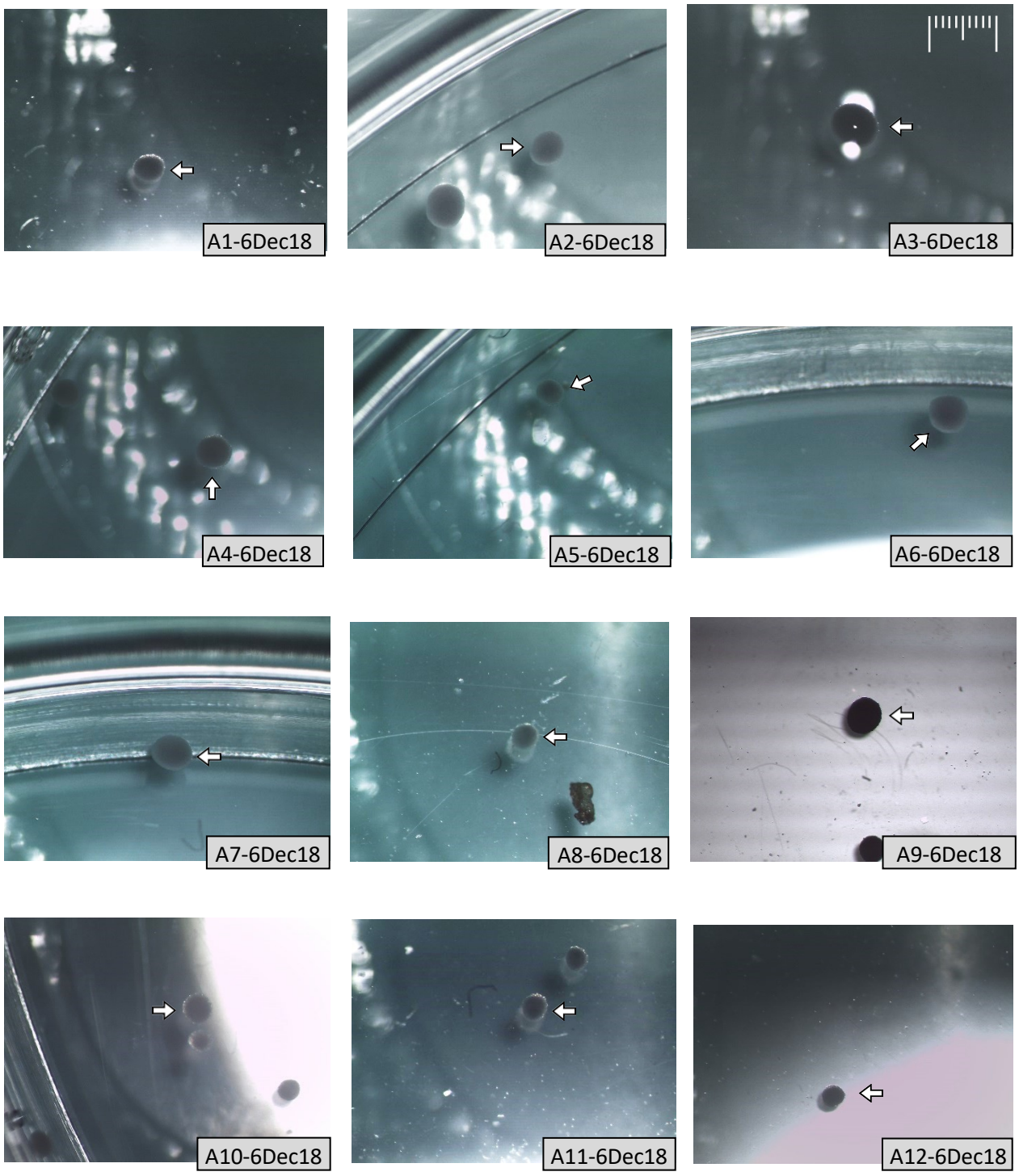


Figure 3: Images of first of four sets of spheroids transferred on 12/06/2018. The transferred spheroid is noted by the white arrow. Each interval of the scale (upper right) indicates 100 µm. In all cases of ovoid spheroids, diameter is estimated by the square root of the product of the lengths of the major and minor axes.

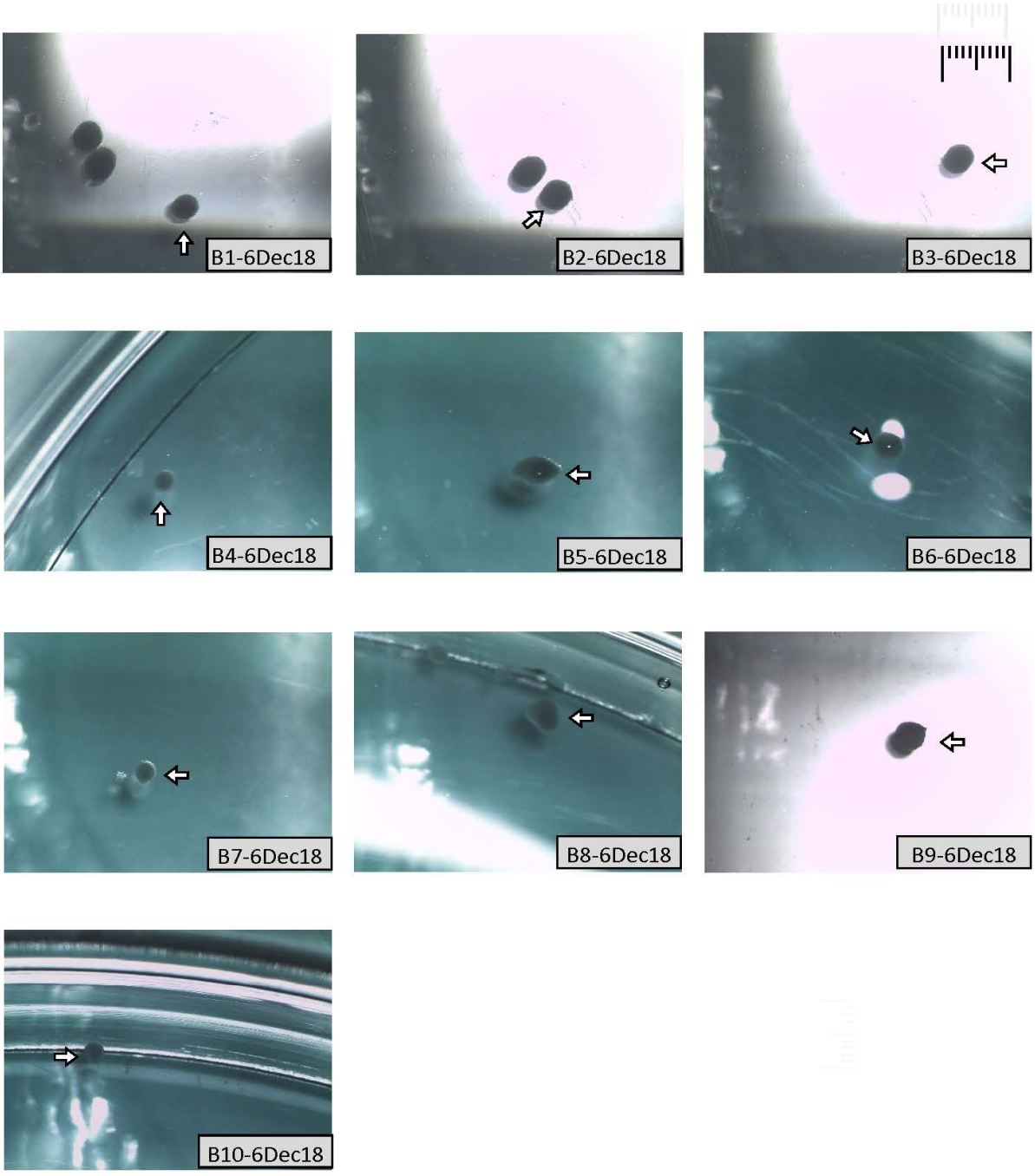


Figure 4: Images of the second of four sets of spheroids transferred on 12/06/2018. The transferred spheroid is noted by the arrow. Each interval of the scale (upper right) indicates 100 µm.

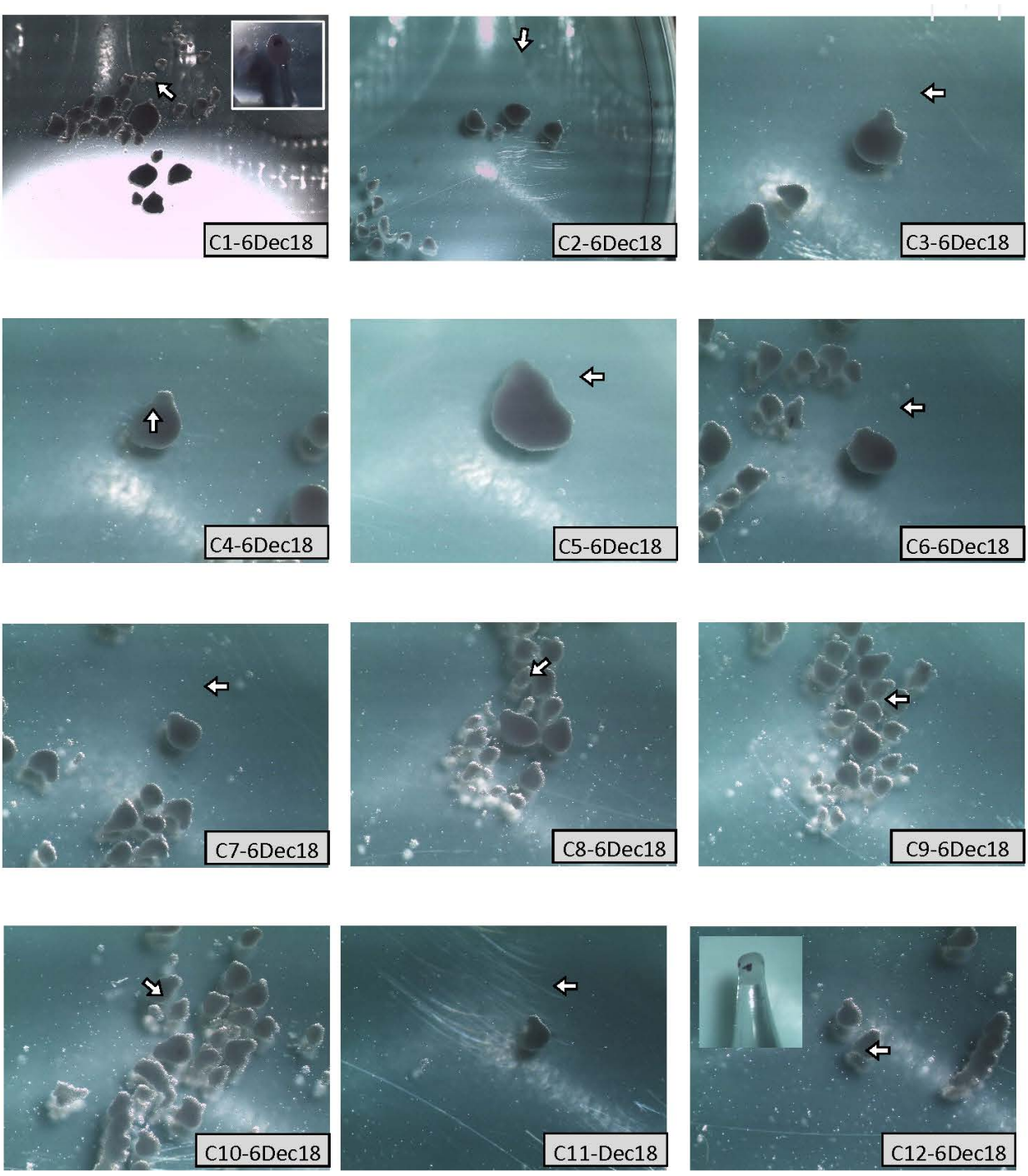


Figure 5: Images of the third of four sets of spheroids transferred on 12/06/2018. The transferred spheroid is noted by the arrow. Each interval of the scale (upper right) indicates 100 µm.

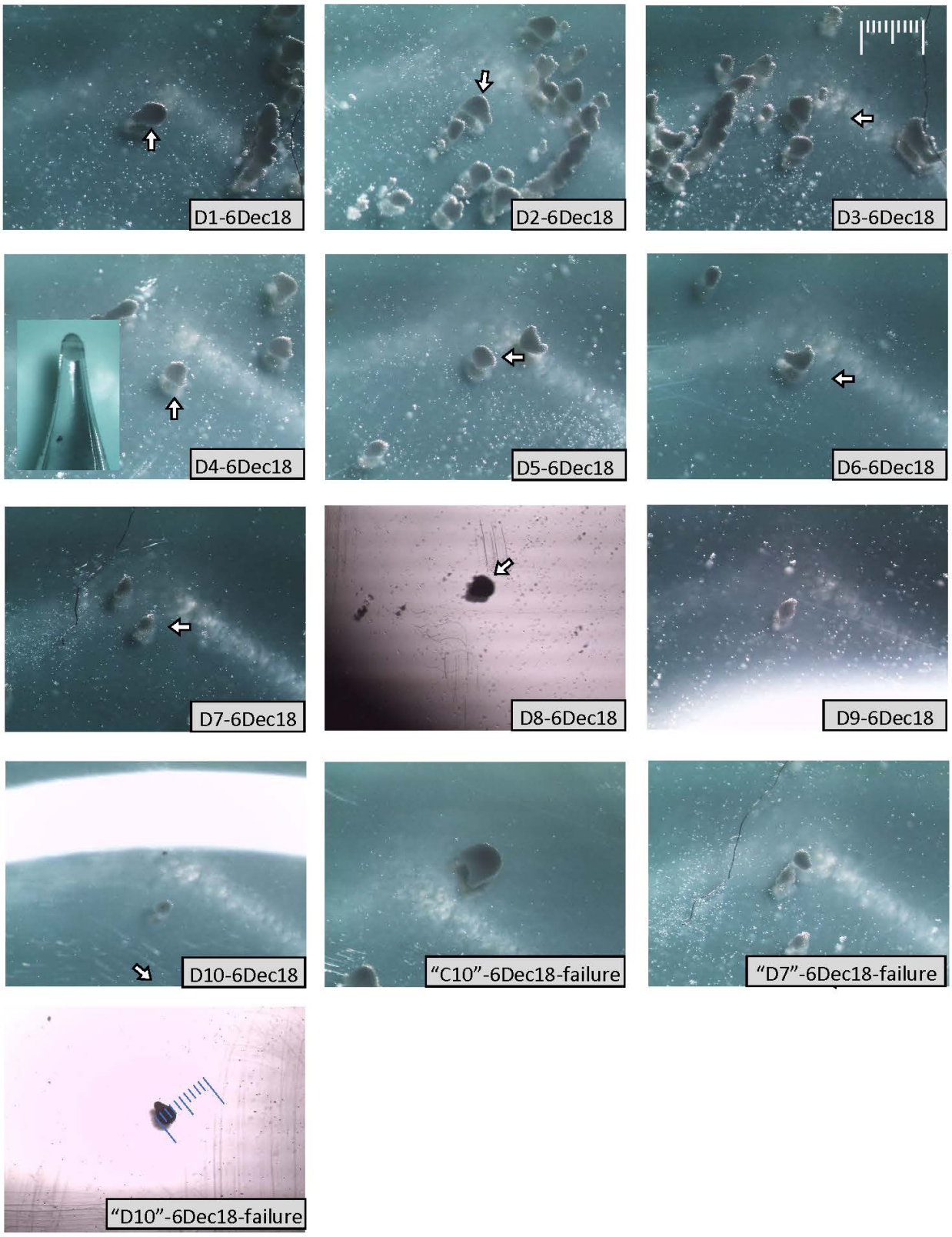


Figure 6: Images of the fourth of four sets of spheroids transferred on 12/06/2018. The transferred spheroid is noted by the arrow. Each interval of the scale (upper right corner).

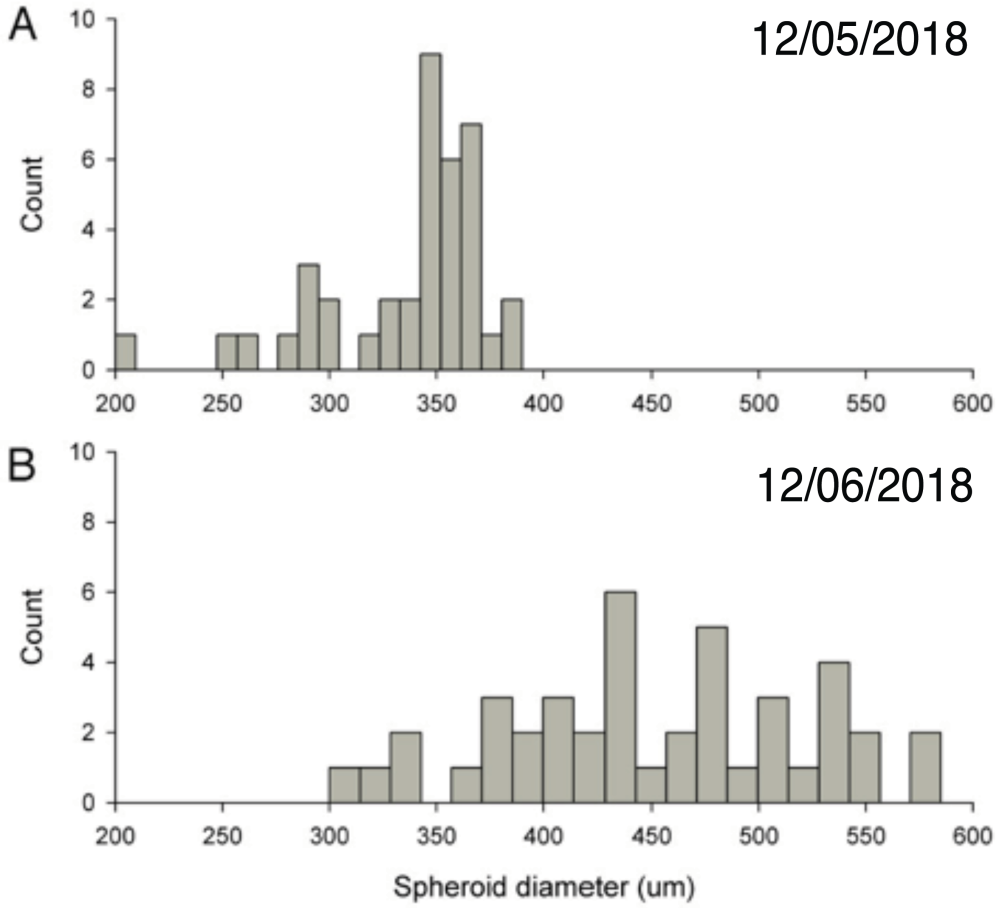


Figure 7: Histogram of diameter of hESC-derived ONP spheroids. (A): Human ESC-derived sephroids transferred on 12/05/2019. (B): Human ESC-derived pheroids transferred on 12/06/2019.

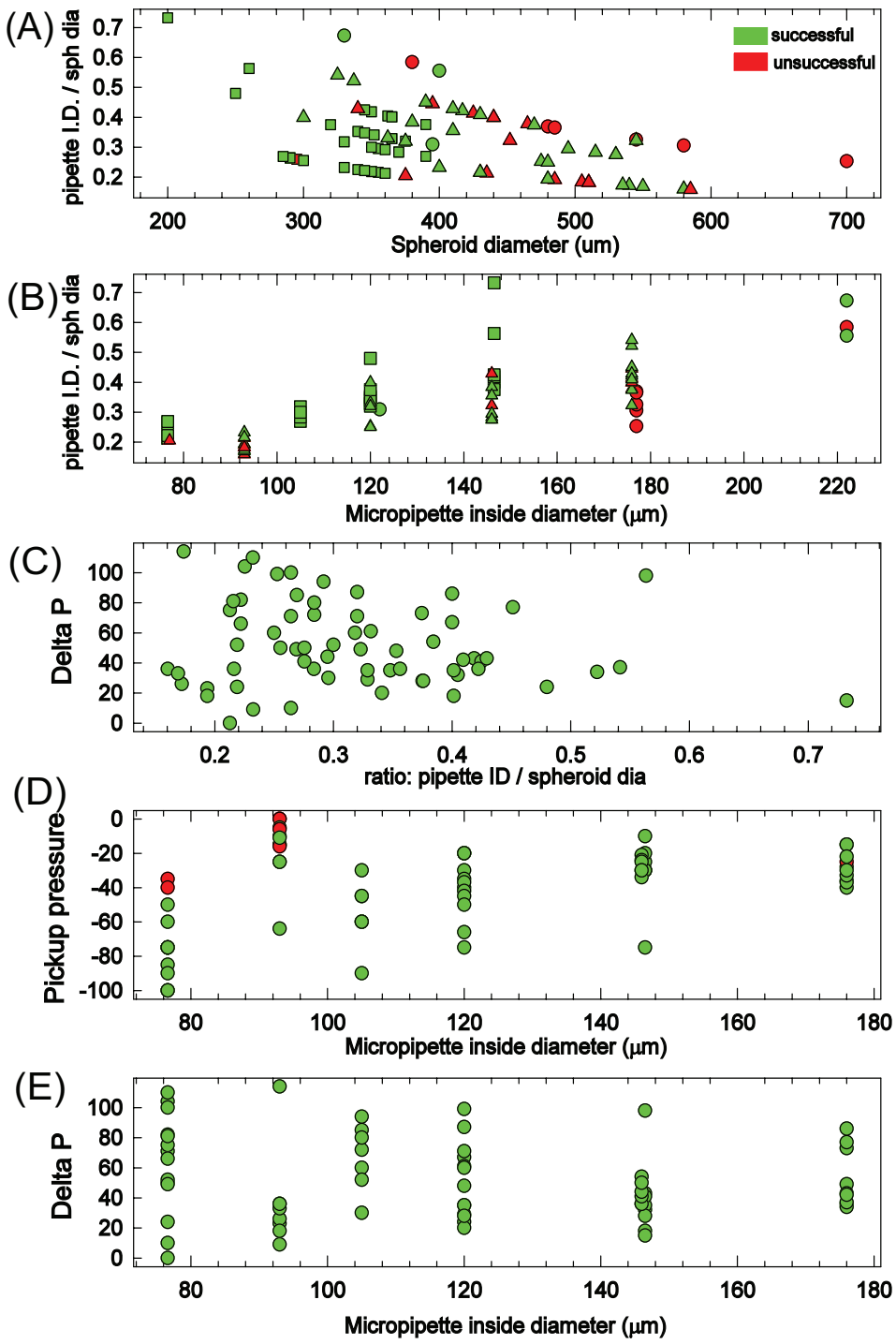


Figure 8: A series of quantitative analysis on hESC-derived ONP spheroid transfer experiments on Dec 5th and Dec 6th, 2018. (A): A scatter plot of pipette I.D. (um)/spheroid diameter (um) in relation to spheroid diameter (um). (B): A scatter plot of pipette I.D. (um)/spheroid diameter (um) in relation to micropipette I.D. (um). (C): A scatter plot of Delta P in relation to micropipette I.D. (um)/spheroid diameter (um). (D): A scatter plot of a pickup pressure of a spheroid in relation to Micropipette I.D. (um). (E): A scatter plot of delta P in relation to Micropipette diameter (um). Note that in figure 2A,2B, and 2D, a green colored dot indicates a "successful" transfer and a red-colored dot indicates a "unsuccessful" transfer.

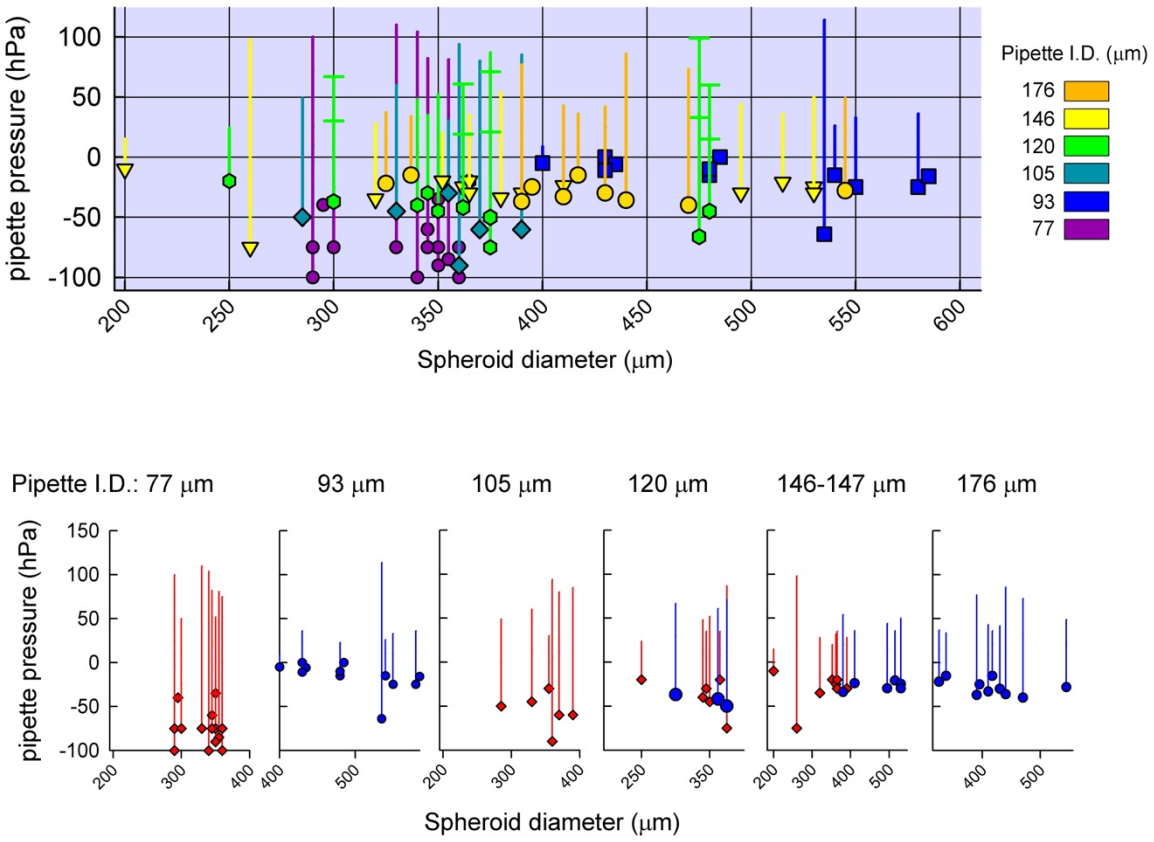


Figure 9: A series of quantitative analysis on hESC-derived ONP spheroid transfer experiments on Dec 5th and Dec 6th, 2018 (Part 2). (A panel on the top): The range of a micro-glass pipette pressure (hPa) that was used to transfer a hESC-derived ONP spheroids in relation to the ONP diameter (um) with 6 different pipette I.D. size (um). (A panel on the bottom): The range of a micro-glass pipette pressure (hPa) in relation to the ONP diameter with 6 different pipette I.D. size (um) that were plotted based on an individual pipette I.D. (um).

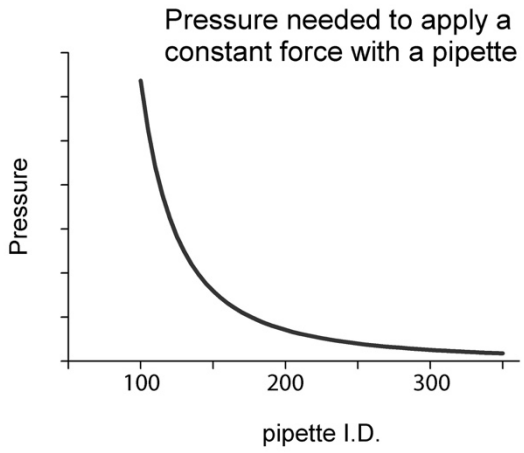


Figure 10: A theoretical model on the relationship between a micropipette pressure (hPa) and a pipette I.D. (um).

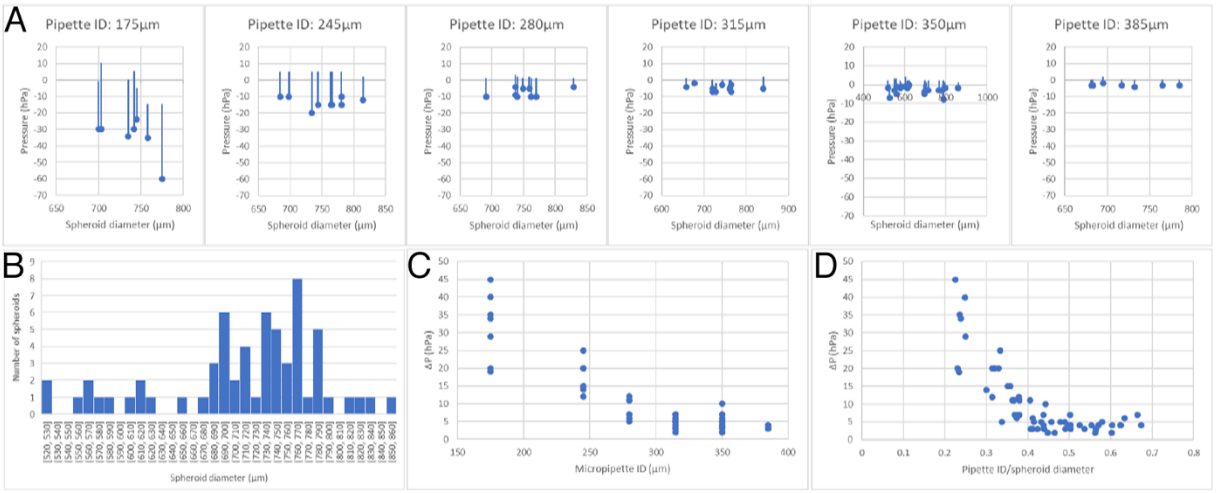


Figure 11: A series of quantitative analysis on hESC-derived ONP spheroid transfer experiments on April 11th, 2019. (A): Capture and release pressures for spheroids by individual pipette used. As pipette inner diameter increases, less negative pressure is required for spheroid capture. (B): Spheroid diameter histogram. Spheroids used range from 520µm to 860µm. (C): Pressure differential. As pipette inner diameter increases, the difference between the spheroid capture and release pressures drops. (D): Pressure differential by pipette/spheroid ratio. The larger the ratio of pipette inner diameter to spheroid diameter, the lower the pressure differential required for spheroid transfer. (Note: (A) is the entire top row).

Based on the data described earlier in this report, we then went on performing 3D spheroid viability assays. We aimed to identify the viability assay with the best performance to optimize the ONP spheroid diameter and a pipette I.D. diameter. For this purpose, we used the LIVE/DEAD Cell Viability Assay (Live Technologies, Carlsbad CA) to evaluate the cytotoxic damage by physical treatment. We first performed positive and negative control. We also evaluate to see if the fixation process effects the result. Cytotoxicity was caused by treating a spheroid with 70% (v/%) ethanol for 30 minutes, using ETOH disrupting cell membrane that results in cytotoxicity. Fixation was performed by 4% PFA for 30 minutes. Spheroids were incubated in first in calcein AM (2uM) followed by ethidium homodimer one (4uM) in PBS for 45 minutes in room temperature. We first obtained a positive control and a negative control to evaluate the reliability of the LIVE/DEAD Cell Viability Assay kit. There were four conditions for this experiment. In the first condition, we treated a GP293 spheroid with 70% (v/%) ethanol for 30 minutes to cause cytotoxicity. Half of these spheroids were then fixed with 4% PFA for 20 mins and the other half of these spheroids were not fixed. In the second condition, we had a live GP293 spheroid with fixed with PFA and without fixing with PFA. Figure 8 shows confocal images of positive control conditions and of negative control conditions. As shown in Figure 12, Figure 11A, B, E, F, G, and H demonstrate expected outcome, however, Figure 3 C indicates false positive (positive in Live assay even though the spheroid was treated with 70% ETOH). We speculated that because of the low objective (20x), individual discrete cells were not imaged, and there are some clumps of cells that appear both live (green) and dead (red) due to the condition of adjacent cells.

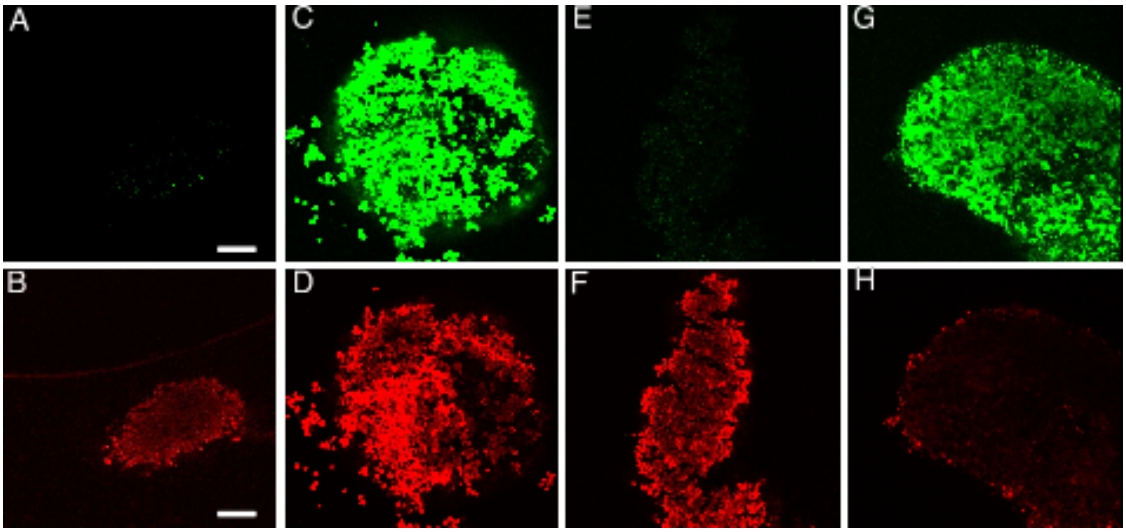


Figure 12: Positive and negative control of the Live and Dead Assay on a GP293 spheroid (The upper row indicates signal in a green channel (LIVE) and the lower row indicates signal in a red channel (DEAD). (A, B): An ETOH-treated & unfixed GP293 spheroid. (C, D): A non-ETOH-treated & unfixed GP293 spheroid. (E, F): An ETOH-treated & fixed GP293 spheroid. (G, H): A non-ETOH-treated and fixed GP293 spheroid. Scale bar = 100 um.

We then performed a Live/Dead Cell Viability Assay (Live Technologies, Carlsbad CA) on twelve hESC-derived ONP spheroids to evaluate our hESC-derived ONPs for the cytotoxic damage by the transfer by a micro-glass pipette. These 12 spheroids were chosen based on data obtained for Figure 4D in that the ratio of a pipette ID to a spheroid diameter were lower that 5 delta Pa. Our hypothesis was that due to the lowest delta Pa would lead into the lowest cell death that could occur during the transfer. The initial analysis revealed that all of 12 ONP spheroids appeared to be alive after the transfer (Figure 13-15). Quantitative analysis will be further performed in the next quarter. We will also perform a Live/Dead Cell Viability Assay on a ONP spheroid in that the ratio of a pipette ID to Spheroid diameter between 0.2 and 0.3. In this range, the delta Pa ranged 20-45 delta Pa; which is much higher than 5 delta Pa. Our hypothesis here is that if we use higher delta Pa, the high delta Pa could potentially trigger an apoptosis cascade, which could result in more cell death on the Live/Dead Cell Viability Assay.

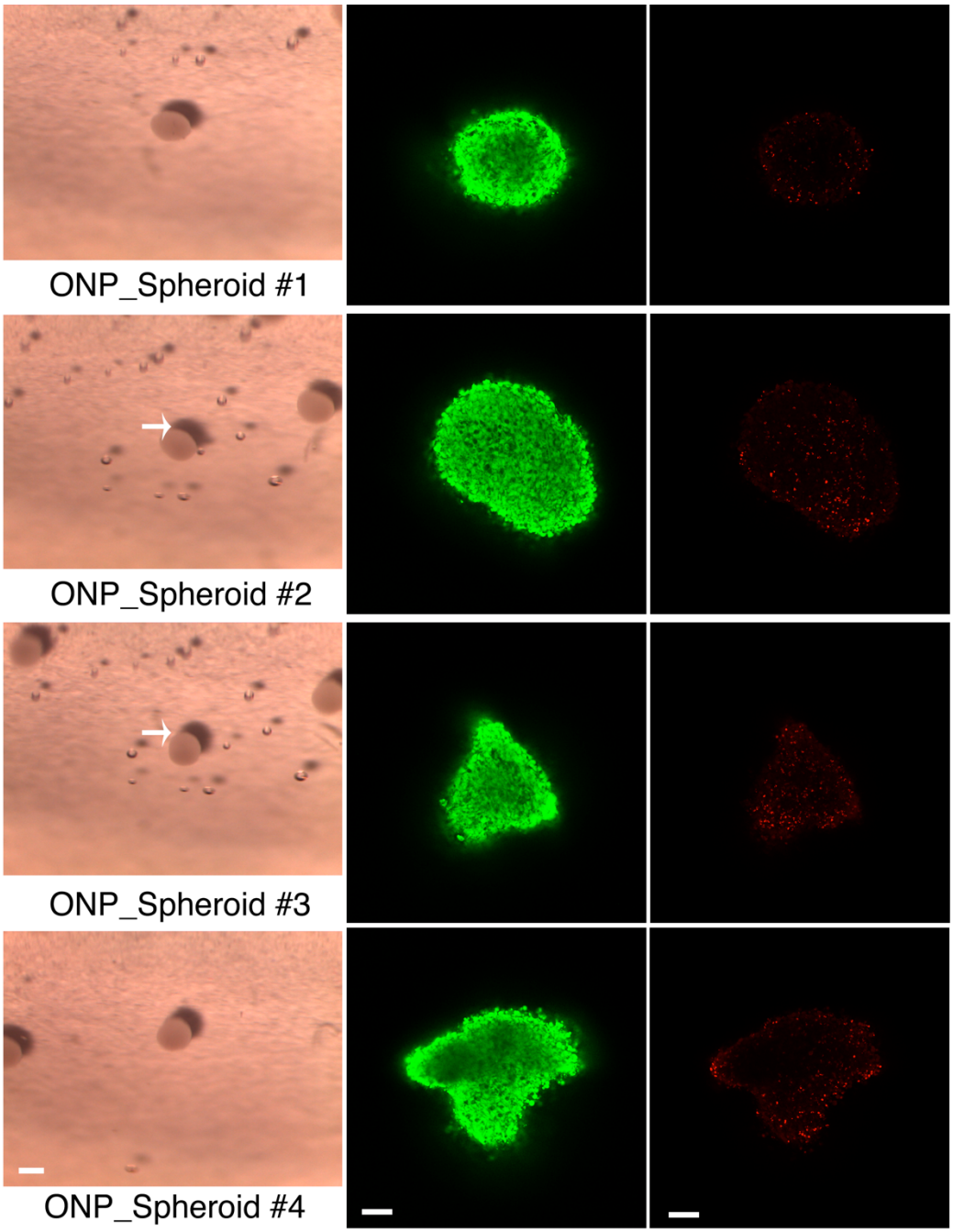


Figure 13: Microscopic images on hESC-derived ONP spheroids (#1-4) (the first column) and a series of confocal fluorescent microscope images of a Live/Dead Cell Viability Assay on the ONP spheroids (green: alive and red: dead. Scale bar for the microscopic image: 500 um. Scale bar for a Live/ Dead Cell Viability Assay: 100 um.

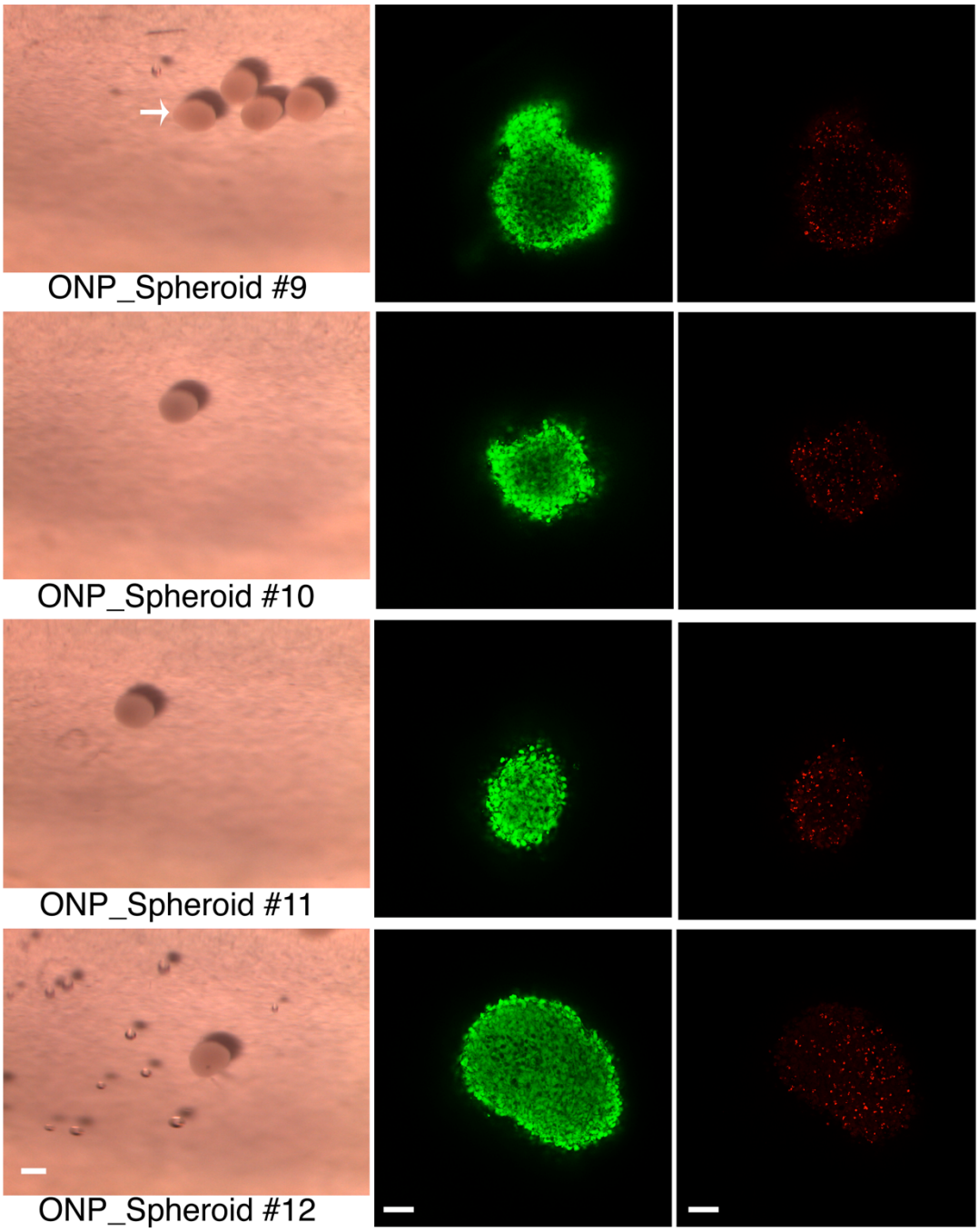


Figure 14: Microscopic images on hESC-derived ONP spheroids (#5-8) (the first column) and a series of confocal fluorescent microscope images of a Live/Dead Cell Viability Assay on the ONP spheroids (green: alive and red: dead). Scale bar for the microscopic image: 500 um. Scale bar for a Live/ Dead Cell Viability Assay: 100 um.

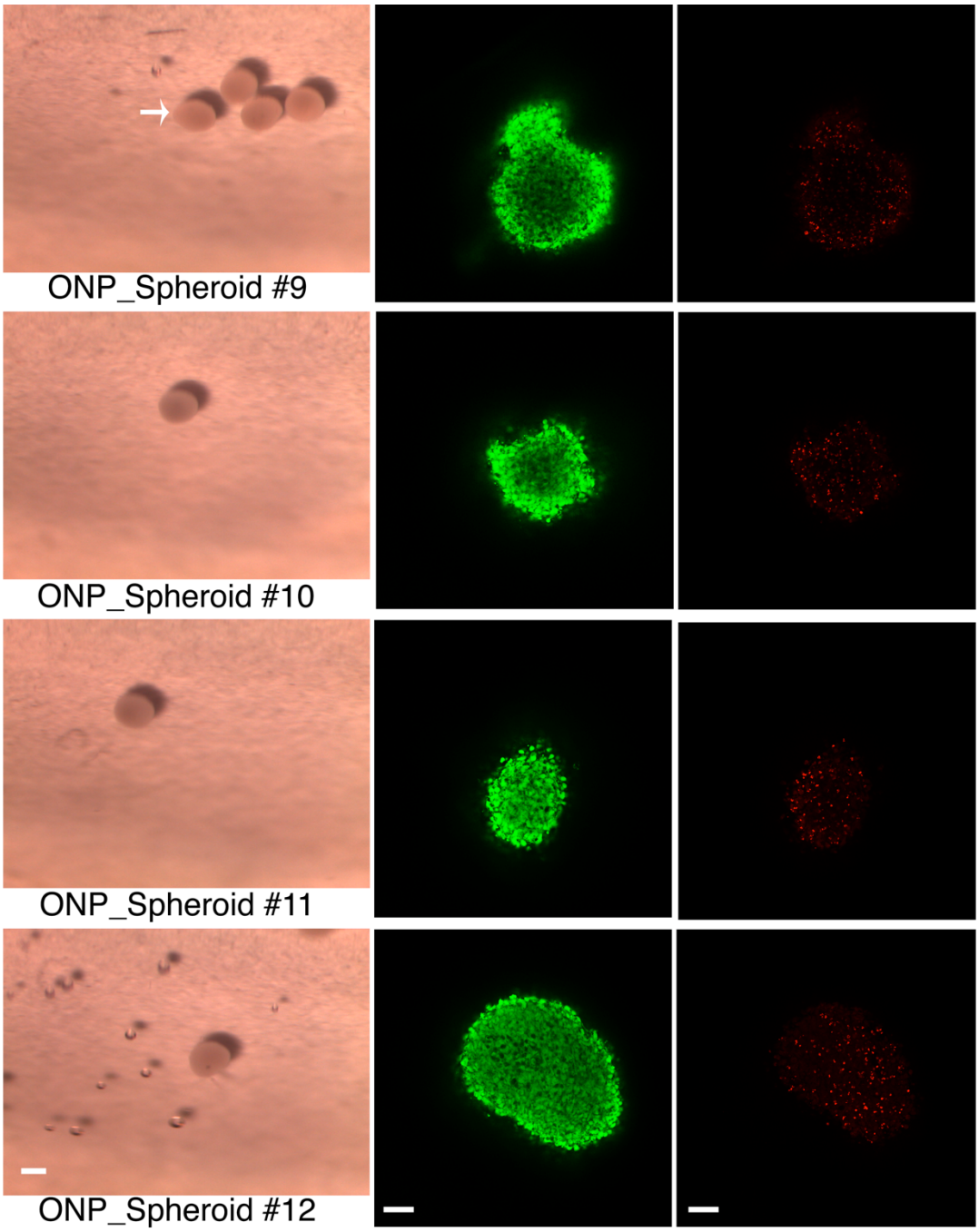


Figure 15: Microscopic images on hESC-derived ONP spheroids (#9-12) (the first column) and a series of confocal fluorescent microscope images of a Live/Dead Cell Viability Assay on the ONP spheroids (green: alive and red: dead). Scale bar for the microscopic image: 500 um. Scale bar for a Live/ Dead Cell Viability Assay: 100 um.

Specific Aim 2: In this aim, we will create a supportive biochemical/biophysical stem cell niches for successful transplantation of hESC-derived 3-D multicellular aggregates into the cochlea. For this purpose, we chose the POlyhedrin Delivery System (PODS) that uses a polyhedron protein crystal to encase and protect a co-expressed cargo protein: Brain Derived neurotrophic Factor (BDNF) to provide a biochemical stem cell niche. In addition, we used NFC hydrogels of GrowDexT®, a biocompatible nanofibril cellulose hydrogel that provides a biophysical stem cell niche. For this purpose, we first started performing 3-D culture of hESC-derived ONPs spheroid using H1, H7, and H9-ESCs. This was carried out based on an already-established protocol can be seen in Figure 1. Generation of hESC-derived ONPs spheroid was performed using both a PrimeSurface® 3D Culture Spheroid plates: Ultra-low Attachment (ULA) Plates (S-Bio, NH) and an EZSphere (Nacalai USA, San Diego, CA).

Spheroid formation and culture with NFC hydrogel

hESC-derived ONPs were generated based on our established protocol (Fig.1).[5] Human ESC-derived late-stage ONPs were passaged to a 96-well Clear Round Bottom Ultra-Low Attachment Microplate (#7007; Corning, NY, USA) at 50,000 cells per well and centrifuged at 900 rpm for 20 minutes followed by incubation at 37˚C for 2 days. Late-stage ONP spheroids were also generated by seeding at 1 million cells per plate into EZSPHERE (#2641701; Nacalai, CA, USA) at 37˚C for 2 days. A 96-well Flat-Bottom μClear plate (Greiner Bio-One, Nurtigen, Germany) was loaded with GrowDex®-T (#200103010; UPM Biomedicals, Helsinki, Finland) pre-mixed with 800,000 PODS Human BDNF (PODS®-hBDNF) (#PPH1-50; Cell Guidance Systems, Cambridge, UK). Spheroids were then carefully transferred from culture plates to the Flat Bottom plate with direct injection into GrowDex®-T solution. GrowDex®-T was diluted to 0.5% (w/v) and 0.375% (w/v) throughout the *in vitro* study based on manufacturer's instructions. Anionic NFC hydrogel (GrowDex®-T) was provided by UPM-Kymmene Corporation (Helsinki, Finland). Cellulose kraft pulp was chemically modified and fibrillated to form anionic NFC hydrogels - the diameter of which ranged from 4–10 nm and length 500–10,000 nm, measured using electron microscopy. PODS®-hBDNF was provided by Cell Guidance System (Cambridge, U.K.).

Immunocytochemistry (3-D spheroid culture)

For hESC-derived late-stage ONP spheroids, culture media were first removed and spheroids were fixed with 4% (w/v) paraformaldehyde followed by blocking overnight in a solution composed of 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) and 0.1% Triton X-100 in Dulbecco's Phosphate-Buffered Saline (Corning, NY, USA). Each sample was incubated for 3 days at room temperature with the following antibodies: anti-beta-III Tubulin (ab18207; Abcam, Cambridge, MA), anti-PAX8 (ab13611; Abcam, Cambridge, MA), anti-MAP2 (1:50, Santa Cruz), anti-VGluT2 (1:500, #AB2251-I; MilliporeSigma, Burlington, MA). This was followed by several PBS washes and incubation in the dark for 3 days at room temperature with Alexa Fluor 488 and 647 (#ab150129, #ab150075; Abcam, Cambridge, UK). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; #D1306; Thermo Fisher Scientific, Waltham, MA) for 30 minutes. Images were then assessed using a Nikon A1 confocal microscope (Tokyo, Japan). ImageJ ver. 2.0.0-rc-69/1.52p. (National Institutes of Health, Bethesda, MD) was used to quantify images.

Enzyme-linked immunosorbent assay (ELISA) for BDNF

To measure the concentration of BDNF secreted from PODS crystals, culture media of both a control and an experimental condition were collected at each time point and immediately stored at -80˚C before running an ELISA. The same method was applied with RhBDNF degradation. All samples were quantified with a BDNF ELISA kit (#BGK23560, PeproTech, NJ) and the results were analyzed with Synergy HTX Multi-Mode Reader (BioTek, VT, USA) at 450nm wavelength as instructed from the kit.

Results:

Differentiation of SGNs in vitro

We first performed immunocytochemical characterization of human ESC-derived (H7) ONP that were treated with BDNF-PODS® and NIM for otic neuronal differentiation (see also Figure 1 for the detailed protocol). Figure 16A shows summary of the protocol for the generation of hESC-derived otic neurons from LONPs. Figure 16B shows a phase-contrast photomicrograph of hESC-derived late-ONPs that had been treated with sonic hedgehog (SHH), all-trans retinoic acid (ATRA), epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), and insulin growth factor-1 (IGF-1) for seven days. Note the typical morphology of hESC-derived ONPs in that a cell has shaped into a "diamond"-like figure; ready to extend neurites. Figure 16C shows a phase-contrast photomicrograph of hESC-derived late-ONPs that has been cultured with PODS® -hBDNF for 7 days. The morphology of hESC-derived late-ONPs indicates a typical bipolar morphology that is consistent with human SGNs.[10] Figure 16D-16J demonstrates immunocytochemical characteristics of hESC-derived late-ONPs that were treated with BDNF-PODS® and NIM. Human hESC-derived late-stage ONPs expressed typical markers for human ONPs that have been previously reported.[11] The makers that the hESC-derived ONPs expressed were GATA3 (otic lineage),[12] PAX8 (otic lineage),[13] NEUROG1 (neuroblast),[14] SOX2 (neuronal progenitor),[15] nestin (neuronal progenitor),[16] VGLUT2 (a glutamatergic neuron),[17] beta-III tubulin (neuron),[18] and peripherin (a peripheral neuron).[19] Cells treated with PODS®-hBDNF significantly decreased the rate of Nestin and PAX8 expression from 60% to 40%, suggesting that the culture with PODS-hBDNF enhanced ONP neuronal differentiation towards SGNs. However, there were no differences in expression of the otic marker PAX8 between RhBDNF and PODS®-hBDNF treatment (Fig. 16K).

Formation of hESCs derived SGN spheroids

To increase cell survival, dissociated ONPs were formed into hESC-derived SGN spheroids using EZSPHERE and 96-well Clear Round Bottom Ultra-Low Attachment Microplate. A live neuron assay was performed to evaluate the viability of hESC-derived SGN spheroids culture in NIM/Brainphys® for 7 days. Spheroids stained with NeuroFluor™ NeuO (#01801; STEMCELL Technologies, BC, Canada) showed great survival of cells during three-dimensional culturing (Fig. 17C). hESC-derived SGN spheroids also expressed neuronal markers PAX8, β-III tubulin, and Nestin when cultured in NIM/Brainphy® (Fig. 17D).

In vitro culture of hESC-derived SGN spheroids with GrowDex-T and PODS®- hBDNF

Before initiating neuronal differentiation with PODS®-hBDNF, hESC-derived SGN spheroids were cultured with 0.375% GrowDex®-T and assessed by staining with PAX8, β-III tubulin, MAP2, VGLUT2, and Peripherin, showing otic neuronal signals (Fig. 18F, G, & H). hESC-

derived ONP spheroids were further cultured with GrowDex®-T and PODS®-hBDNF for 7 days. Samples were then stained with PAX8 and β-III tubulin (Fig. 18C-F&19A-D). Quantification of immunocytochemistry implies that spheroids treated with RhBDNF and PODS®-hBDNF showed a significantly lower rate of PAX8 expression when cultured with both 0.5% and 0.375% GrowDex®-T (Fig. 19E). Moreover, figure 19G reveals that the presence of GrowDex®-T increased the number of SGN-like cells at a percentage higher than 90%. Spheroids cultured with only NIM showed limited evidence of neurite extension (Fig. 18C&19A). However, PODS®-hBDNF significantly enhanced the growth of neurite length: cells cultured in both 0.5% and 0.375% GrowDex®-T had neurites about twice as long in PODS®-hBDNF than in RhBDNF and five times longer than just GrowDex®-T (Fig. 19F). PODS®-hBDNF also increased SGNs' branching abilities with the increment of total length, with higher arborization observed in PODS®-hBDNF than in three other controls; a significant augment was also noticed when culturing in 0.375% GrowDex®-T (Fig. 19H).

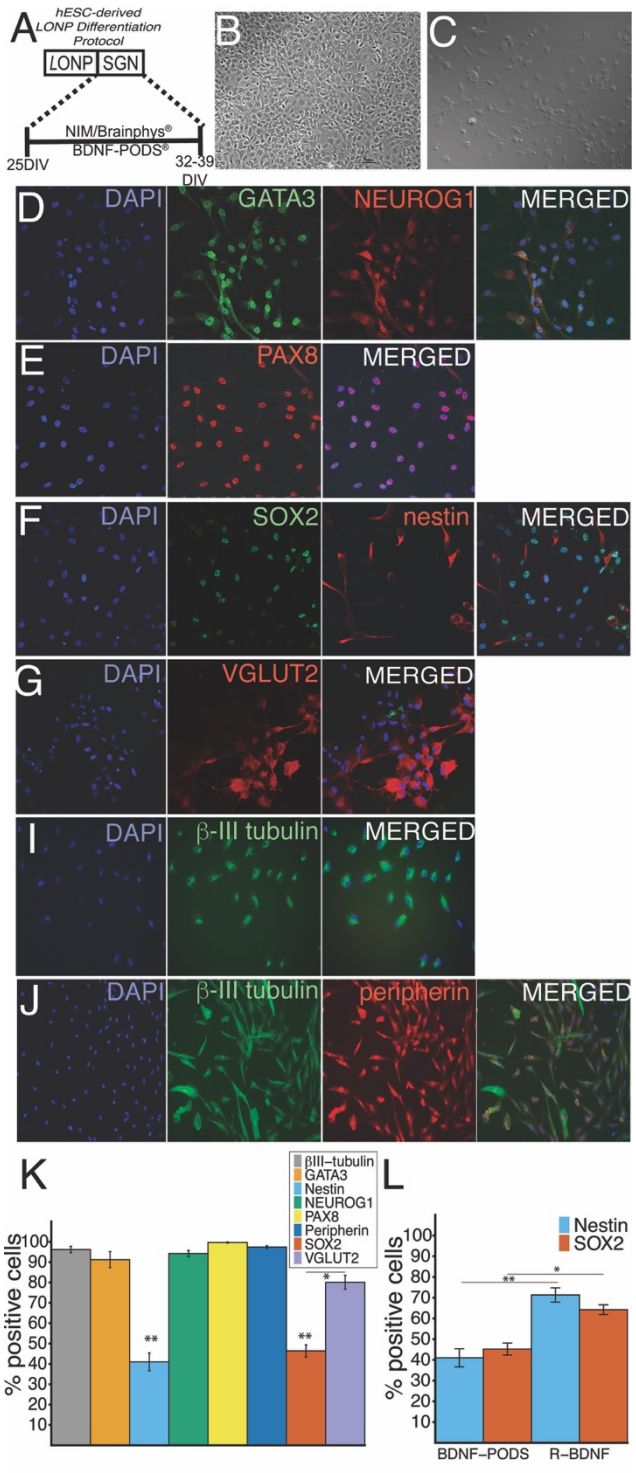


Figure 16: Assessment of induction of spiral ganglion neuron (SGN)-like glutamatergic neurons from late-stage ONPs. **(A):** Stepwise treatment for SGN induction. On day 25, PODS®-hBDNF was started in NIM for 7-14 days. D: days. **(B):** Phase-contrast photomicrographs of ONPs after SHH treatment. **(C):** Phase-contrast photomicrographs of SGNs. **(D)(E)(F) (G)(H)(I):** Immunocytochemistry of late-stage ONPs treated with PODS®-hBDNF in NIM/Brainphys® shows expression of neuronal markers Nestin, GATA3, β-III tubulin, VGLUT2, peripherin, NEUROG1, PAX8 and SOX2. **(J):** Quantification of all the neuronal markers for positivity % (n = 3). **(K):** Quantification of PODS®-hBDNF and RhBDNF treated cells with nestin, PAX8 and SOX2. *p<0.05, ** $p < 0.01$ by one-way ANOVA with Tukey's post-hoc test.

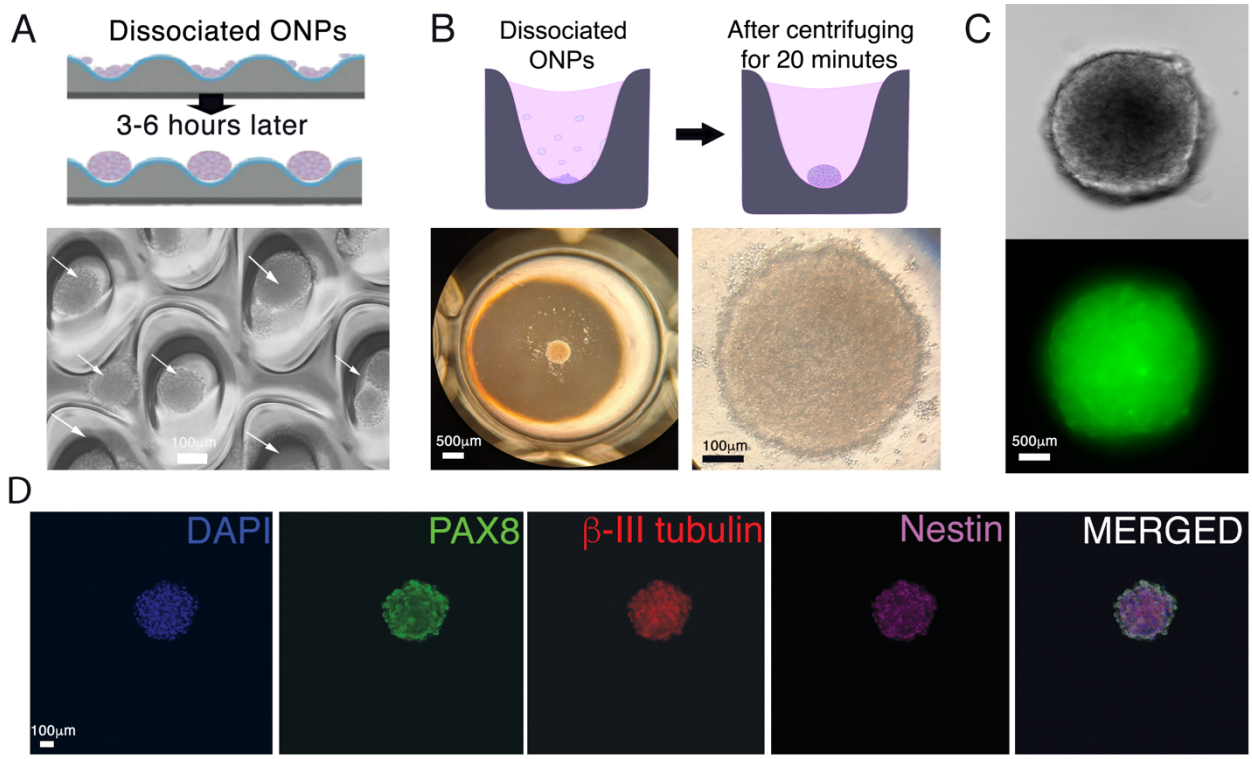


Figure 17: Formation of hESCs derived SGNs spheroids. **(A):** Schematic figure of forming hESCs derived ONPs spheroids using EZSHPERE plate and phase-contrast of spheroids within the plate. **(B):** Schematic figure of forming hESCs derived ONPs spheroids using 96-well Clear Round Bottom Ultra-Low Attachment Microplate and phase-contrast of spheroids within the plate. **(C):** Confocal image stained with NeuroFluor^{TM} NeuO. **(D):** Confocal image of hESCs derived ONPs spheroid stained with Nestin, β-III tubulin and PAX8.

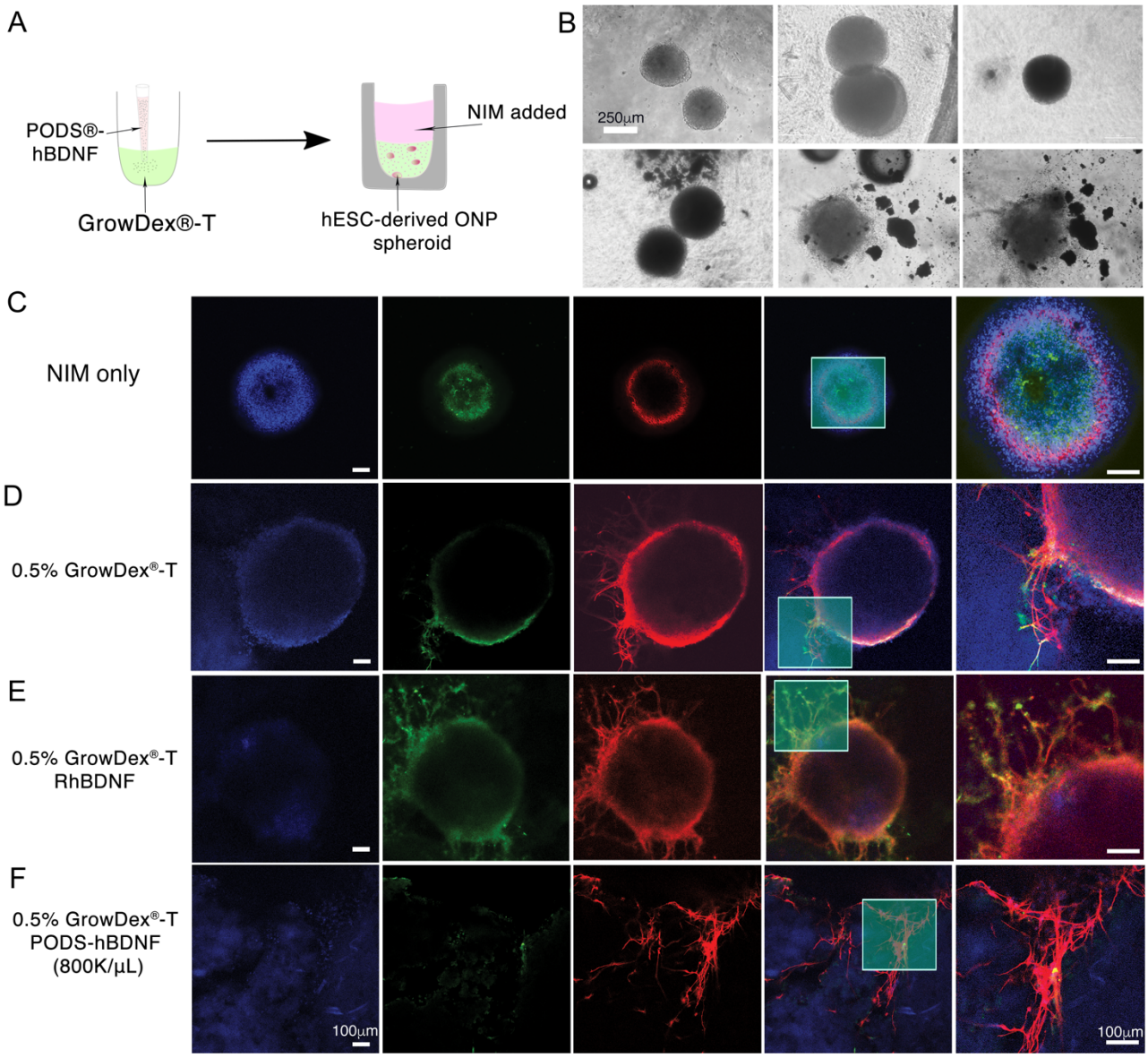


Figure 18: 0.5% GrowDex®-T with hESCs derived ONPs spheroids *in vitro*. **(A):** Schematic figure of PODS®-hBDNF and GrowDex®-T mixture with hESCs derived SGNs spheroids *in vitro*. **(B):** Phase-contrast of hESCs derived ONPs spheroids culture with PODS®-hBDNF and GrowDex®-T mixture. **(C):** Immunocytochemistry of hESCs derived ONPs spheroids culture in NIM for 7 days. **(D):** Immunocytochemistry of hESCs derived ONPs spheroids culture in 0.5% GrowDex®-T for 7 days. **(E):** Immunocytochemistry of hESCs derived ONPs spheroids culture in 0.5% GrowDex®-T with 20 ng/mL of RhBDNF for 7 days. **(F):** Immunocytochemistry of hESCs derived ONPs spheroids culture in 0.5% GrowDex®-T with 800,000 PODS®-hBDNF for 7 days.

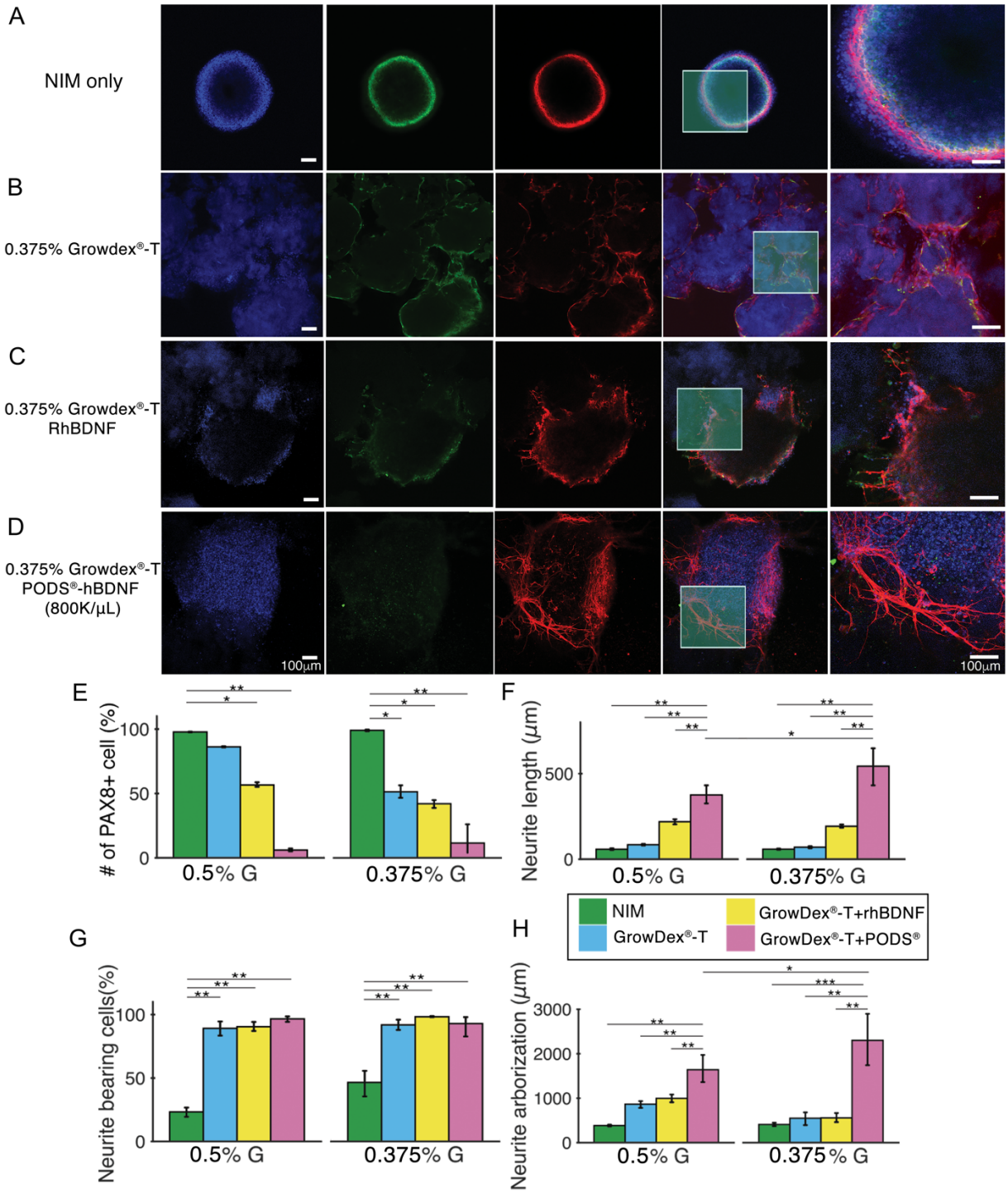


Figure 19: 0.375% GrowDex®-T with hESCs derived ONPs spheroids *in vitro*. (A): Immunocytochemistry of hESCs derived ONPs spheroids culture in NIM for 7 days. (B): Immunocytochemistry of hESCs derived ONPs spheroids culture in 0.375% GrowDex®-T for 7 days. (C): Immunocytochemistry of hESCs derived ONPs spheroids culture in 0.375% GrowDex®-T with 20 ng/mL of RhBDNF for 7 days. (D): Immunocytochemistry of hESCs derived ONPs spheroids culture in 0.375% GrowDex®-T with 800,000 PODS®-hBDNF for 7 days. (E): Quantification of PAX8 positive cells. (F): Quantification of neurite length. (G): Quantification of neurite bearing analysis. (H): Quantification of neurite arborization analysis. *p<0.05, ** $p < 0.01$, $p < 0.001$ by one-way ANOVA with Tukey's post-hoc test.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Kevin Nella (Research Affiliate): Kevin has been accepted for Medical Research Scholars Program at National Institute of Health. Kevin has been working on a quantitative finite element model on diffusion kinetics of BDNF in vitro and also in vivo on this project. Kevin is planning to continue working on the quantitative aspect of Regenerative Medicine in the inner ear at the NIH.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Walk 4 Hearing (Hearing Loss Association on America), Chicago, September 29, 2019.

Discussed future treatment options such as stem-cell replacement therapy in the inner ear and also our design of "bio-hybrid cochlear implant.

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

I would like to continue involvement in Walk 4 Hearing. I am also planning to give a lecture at American Tinnitus association, Chicago branch regarding emerging treatment of sensorineural hearing loss.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to report

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Postdoctoral fellow will be starting her work on this project on 01/04/2020. It has been delayed due to her visa issue, which has been resolved.

A replacement of Key personnel will be also hired on 01/4/2020 provided that his visa will be approved in a timely fashion.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Delay in hiring aforementioned two individual had a significant impact on expenditure, however, both of them will be hired by January, 2020.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Authors: Hsiang-Tsun Chang, MS[1], Andy M. Oleksijew, BS[1], Kyle S. Coots, BS[1], Rachel A. Heuer, BS[1], Kevin T. Nella, BS[1], Christian B. Roque, BS[1], Tammy L. McGuire, BS[2], Akihiro J. Matsuoka, M.D., D.M.Sc., Ph.D.[1,3,4]
Title: *An Engineered three-dimensional Stem Cell Niche in the Inner Ear with Nanofibrillar Cellulose Hydrogel and a Polyhedrin Delivery System*
Journal: Tissue Engineering A
Status of publication: Submitted
Acknowledgement of federal support: yes

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

CONFERENCE PAPERS

1. ISSCR (International Society of Stem Cell Research) 2019, Los Angeles, CA, USA. June 26th-29th, 2019.

W-2106 - CELL DELIVERY VEHICLE FOR HUMAN EMBRYONIC STEM-CELL REGENERATION THERAPY IN THE INNER EAR: UTILIZATION OF HYDROGEL-ENCAPUSULATED OTIC NEURONAL PROGENITOR SPHEROIDS
Wed, Jun 26 - 7:30 PM – 8:30 PM
Akihiro J. Matsuoka* – Northwestern University, United States
Category: Stem Cell Niches

T-2104 - DESIGNING A BIOLOGICAL INTERFACE COMBINING A COCHLEAR IMPLANT WITH HUMAN EMBRYONIC STEM CELLS
Thu, Jun 27 - 7:00 PM – 8:00 PM
Hsiang-Tsun Chang*– Northwestern University, Chicago, IL
Category: Stem Cell Niches

T-2109 - EFFECTS OF LONG-TERM NEUROTROPHIN SIGNALING ON HUMAN

EMBRYONIC STEM CELL-DERIVED SPIRAL GANGLION-LIKE NEURONS
Thu, Jun 27 - 6:00 PM – 7:00 PM
Kevin Nella* – University of Miami, Miami, FL
Category: Technologies for Stem Cell Research

F-4068 - PLANT-DERIVED CELLULOSE HYDROGEL AS A MATRIX FOR 3D HUMAN STEM CELL PROLIFERATION AND DIFFERENTIATION
Fri, Jun 28 - 7:00 PM – 8:00 PM
Jane Spencer-Fry – UPM-Kymmene Corporation, Helsinki, Finland

2. COMSOL Conference 2019 BOSTON, Boston, MA. October 2nd-5th, 2019.

A COMSOL Multiphysics® Finite Element Model of the Diffusion Profile of Brain Derived Neurotrophic Factor: Biological Validation through a Microfluidics Device
Kevin Nella* – University of Miami, Miami, FL

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

  Nothing to report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

  Nothing to report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*

- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name:	Akihiro Joseph Matsuoka, M.D., D.M.Sc., Ph.D. FACS
Project Role:	Principal Investigator
Researcher Identifier:	https://orcid.org/0000-0002-1066-6514
Nearest person month worked:	3.0
Contribution to Project:	Dr. Matsuoka has performed the overall planning of the project, defining experimental directions and designs, interpreting data, communicating with his co-investigator, statistical analysis, and reporting project results to the sponsor and the scientific community in the form of progress reports, papers, and presentations.

Name:	Charles A. Miller, Ph.D.
Project Role:	Senior Research Associate
Researcher Identifier:	N/A
Nearest person month worked:	3.6
Contribution to Project:	Dr. Miller has performed work in 3-D aggregate transplantation using micropipettes equipped Sutter microinjection system as well as a World Precision Instruments volume-controlled injector.

Name:	Andrew M. Oleksijew, B.S.
Project Role:	Research Technician
Researcher Identifier:	N/A
Nearest person month worked:	6
Contribution to Project:	Mr. Oleksijew has performed work in nanofibrillar cellulose gel generation, incorporation of POS crystals into Geltrex, and immunohistochemistry.

Name:	Rachel M. Hauer, B.S.
Project Role:	Research Technician
Researcher Identifier:	N/A
Nearest person month worked:	6
Contribution to Project:	This technician performed a Live-Dead cell assay, nanofibrillar cellulose gel generation, incorporation of POS crystals into Growdex, and immunohistochemistry.

Name:	Trent Chang, M.S.
Project Role:	Research Technician
Researcher Identifier:	N/A
Nearest person month worked:	6
Contribution to Project:	This technician performed a Live-Dead cell assay, nanofibrillar cellulose gel generation, incorporation of POS crystals into Growdex, and immunocytochemistry.

Name:	Kevin Nella, B.S.
Project Role:	Research Scholar (volunteer medical student)
Researcher Identifier:	N/A
Nearest person month worked:	6
Contribution to Project:	This individual performed nanofibrillar cellulose gel generation, incorporation of POS crystals into Growdex.
Name:	Christian Roque
Project Role:	Research Technician
Researcher Identifier:	N/A
Nearest person month worked:	12
Contribution to Project:	This technician performed a Live-Dead cell assay, nanofibrillar cellulose gel generation, incorporation of POS crystals into Growdex, and immunocytochemistry.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Stem Cell Regeneration of Human Spiral Ganglion Neurons toward Hearing Restoration

PI: Akihiro J. Matsuoka, M.D., D.M.Sc., Ph.D.
Co-Investigator: Eri Hashino, Ph.D.
Award: $1,420,283

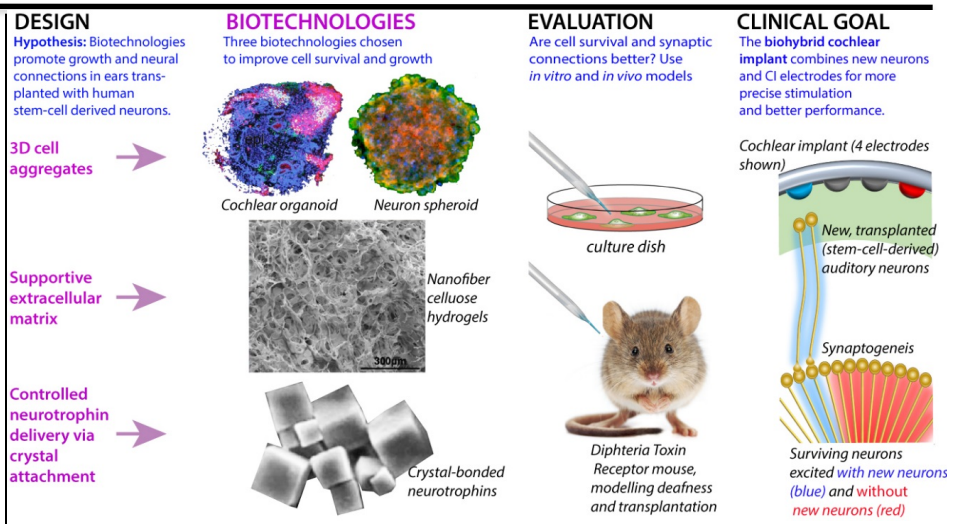
Org: Northwestern University Feinberg School of Medicine (NU)
Indiana University School of Medicine (IUSM: consortium)

Study Aims

- Optimize the delivery of human stem-cell-derived auditory neurons to the cochlea.
- Assess how well three bio-engineered support strategies improve survival and growth of those neurons using an *in vitro* (culture dish) test bed.
- Refine the results by assessing the strategies in an animal model. Use a genetically engineered mouse (providing a refined deaf-cochlea model) to transplant stem-cell-derived neurons and the support strategies into the deaf cochlea.
- Key outcome measures include cell survival, neurite growth, auditory neuronal differentiation, and synapse formation.

Approach

- Two cell-delivery methods (based on controlling pressure or flow) will be tested.
- Use spheroids and organoids, both containing stem-cell-derived neurons, to assess the hypothesized positive effects of grouping cells into 3-D aggregates.
- Use our proven method for converting human embryonic stem cells to neurons.
- Also assess the supportive effects of an extracellular support matrix and a neurotrophin, i.e., a protein known to support neuron health.
- The *in vitro* model provides optimization of neurotrophin concentration.
- The *in vivo* model provides study of cell survival, neuronal differentiation, and synapatic plasticity across time.



Accomplishments: (1) Published protocol to direct stem cells to auditory neuron fate
(2) Evaluated extracellular matrix to support neuron survival (manuscript submitted)
(3) Successful cochlear implantation of stem-cell-derived neurons and spheroids

Timeline and Cost

Major Activities		2018	2019	2020	2021
Aim 1 & 2 Generation of inner-ear organoids (IUSM) and neuronal spheroids (NU)					
Aim 1 Optimize cell transplantation methods					
Aim 2 Generation of BRN3A 2A-ntdTomato reporter hESC line (IUSM)					
Aim 2 Assess *in vitro* niche effectiveness					
Aim 2 Assess *in vivo* niche effectiveness					
Data dissemination	Meetings		TERMIS ISSCR COMSOL	ISSCR DOD TERMIS	ISSCR
	Publication prep/submit				
Estimated Budget ($1M)		0.10 M	0.30M	0.30M	0.30M

Updated: Oct 15th, 2019

Goals / Milestones

CY18 Goal – Optimize cell transplantation and begin *in vitro* testing
- ☑ Perform LIVE/DEAD assay to optimize cell transfer techniques
- ☑ Begin *in vitro* evaluation of support strategies (niches) for derived neurons

CY19 Goals – *In vitro* & *in vivo* evaluation of supportive niches
- ☑ Continue *in vitro* evaluations begun in CY18
- ❑ Optimize BDNF (neurotrophin) concentrations for use with *in vivo* model.
- ☑ Begin *in vivo* studies of transplanted neuron survival, neurite growth, and synaptic connectivity using the genetically engineered "DTR" animal model

CY20 Goal – Continue evaluations using DTR mouse model
- ❑ Presentation to DoD

CY21 Goal – Completion of work: Results characterization
- ❑ Final assessments: neuron survival, neurite growth, auditory neuronal differentiation, and synaptogenesis
- ❑ Final assessment: effects of post-implantation time on neuron survival

Comments/Challenges/Issues/Concerns
- N/A

Budget Expenditure
Projected Expenditure: $1,420,283
Actual Expenditure: $ 199.318.59 (09/29/2019)