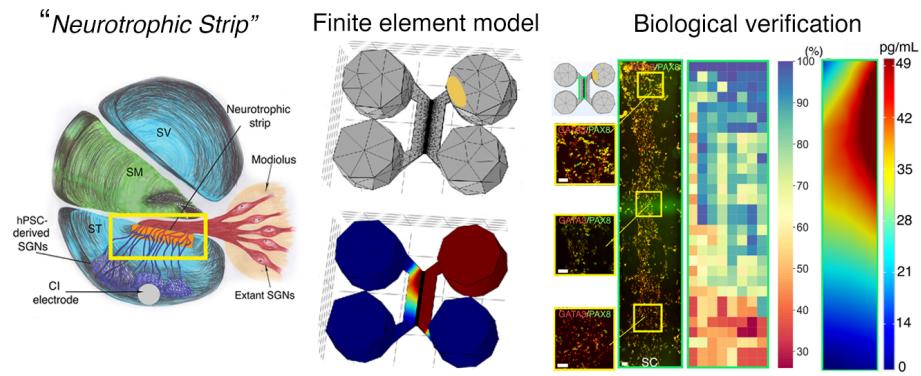


# Graphical Abstract

## Developing the Bioactive Cochlear Implant: Bridging the electrode–neuron gap through neurite guidance

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Christian B. Roque, Akihiro J. Matsuoka



# Developing the Bioactive Cochlear Implant: Bridging the electrode–neuron gap through neurite guidance

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## Abstract

Although cochlear implant (CI) technology has allowed partial restoration of hearing over the last few decades, persistent challenges remain - including the deciphering of rich acoustic signals into a digital pulse-train signal. Among these challenges, the “electrode–neuron gap” poses the most significant obstacle to advancing past the current plateau in CI performance. Resulting issues include limited performance in noisy environments and a poor ability to decode intonation and music. We propose the development of a “neurotrophic strip”—a biological interface that doubly preserves endogenous spiral ganglion neurons (SGNs) while precisely directing the growth of neurites arising from transplanted human pluripotent stem cell (hPSC)-derived otic neuronal progenitors (ONPS), toward endogenous SGNs. We hypothesized that the Polyhedrin Delivery System (PODS®-human brain-derived neurotrophic factor [BDNF]) could stably

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provide an adequate BDNF gradient to hPSC-derived ONPs, thereby simultaneously facilitating otic neuronal differentiation and directional neurite outgrowth. To test this hypothesis, we first devised a finite element model to simulate the *in vitro* BDNF gradient generated by PODS®. For biological verification, we validated the concept of the neurotrophic strip by using a multi-chamber microfluidic device, which mimics the *in vivo* micro-environment more accurately than conventional laboratory plates in terms of volume and concentrations of endogenous/exogenous factors. We were able to generate the optimal BDNF concentration gradient to enable survival, neuronal differentiation toward hPSC-derived SGNs, and directed neurite extension of hPSC-derived SGNs. The technique will allow us to further advance our concept of the “neurotrophic strip” by controlling neurite direction of transplanted hPSC-derived ONPs. This proof-of-concept study provides a step toward the next generation of CI technology.

*Keywords:* human pluripotent stem cells, finite element model, microfluidic device, neurotrophic factor, cochlear implant, neuroelectric interface, stem cell niche, spiral ganglion neurons

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<sup>1</sup> **1. Introduction**

<sup>2</sup> The cochlear implant (CI), which provides functional restoration in patients  
<sup>3</sup> with sensorineural hearing loss, forms a neuro-electronic interface with the pe-  
<sup>4</sup> ripheral auditory nervous system [1]. CI technology functions by electrically  
<sup>5</sup> stimulating the extant population of auditory neurons (i.e., spiral ganglion neu-  
<sup>6</sup> rons [SGNs]). Although CI technology has allowed partial restoration of hearing  
<sup>7</sup> for this patient population over the last few decades, persistent challenges, in-  
<sup>8</sup> cluding the deciphering of rich acoustic signals into digital pulse-train signals,  
<sup>9</sup> remain. Among these challenges, the “electrode-neuron gap” poses the most  
<sup>10</sup> significant obstacle to advancing past the current plateau in CI performance.  
<sup>11</sup> This phenomenon symptomatically manifests as limited performance in noisy  
<sup>12</sup> environments and poor ability to decode intonation and music [2], arguably de-  
<sup>13</sup> creasing quality of life. The gap exists between the CI electrode and the target  
<sup>14</sup> membranes of dendrites in surviving endogenous SGNs [3]. It results in the  
<sup>15</sup> requirement of larger CI excitation fields, leading to current spread that excites  
<sup>16</sup> and therefore disables the neighboring electrodes, resulting in fewer information  
<sup>17</sup> channels to the brain, all within discrete time steps [2, 4]. This can develop into  
<sup>18</sup> a vicious cycle as fewer information channels to the brain also prompt the need  
<sup>19</sup> for larger CI excitation fields. The length of the gap generally spans hundreds of  
<sup>20</sup>  $\mu\text{m}$  [5, 6]. Hahnewald et al. demonstrated *in vitro* that energy needed to elicit  
<sup>21</sup> a response can be reduced by up to 20% by reducing the distance from 40 to  
<sup>22</sup> zero  $\mu\text{m}$  (by growing early postnatal mouse SGN explants on a microelectrode  
<sup>23</sup> array) [4].

<sup>24</sup> Previous work has introduced the concept of a “bioactive” CI to resolve the  
<sup>25</sup> electrode-neuron gap *in vivo*[7, 8, 9]. The bioactive CI combines the current  
<sup>26</sup> state-of-the-art CI technology with emerging stem cell-replacement therapy in  
<sup>27</sup> the inner ear. In this scheme, transplanted human pluripotent stem cell (hPSC)-  
<sup>28</sup> derived SGNs bridge the gap between the CI electrode and surviving endogenous  
<sup>29</sup> SGNs. Furthermore, introducing neurotrophin gradients has been shown to  
<sup>30</sup> guide hPSC grafts in spinal cord injury [10], direct growth of endogenous SGNs

31 toward CI electrodes in the scala tympani [11], and enable transplanted hPSC  
32 derived otic neuronal progenitors (ONPs) to grow neurites toward the modio-  
33 lus [9]. Although promising, these studies failed to observe adequate directed  
34 neurite outgrowth toward endogenous SGNs (i.e., lack of synaptic connections  
35 between hPSC grafts and endogenous SGNs), presumably preventing significant  
36 improvements in functional recovery of hearing.

37 To confront this issue, we propose the development of a “neurotrophic strip”—a  
38 biological interface that doubly preserves endogenous SGNs and precisely directs  
39 the growth of neurites arising from transplanted hPSC-derived ONPs toward  
40 the endogenous SGNs. The highlighted yellow-square area in Figure 1A shows  
41 a schematic diagram of this concept. Here, the neurotrophic strip (shown as  
42 an orange rectangle in Figure 1A) stimulates neurite outgrowth from both the  
43 hPSC-derived ONPs and the endogenous SGNs via a neurotrophic factor gra-  
44 dient [12]. While the concept of using a neurotrophin gradient for directional  
45 axonal growth has existed for a few decades, incorporation of neurotrophin gra-  
46 dients with any tissue- or bio-engineered scaffold has been extremely challenging  
47 due to the lack of self-sustaining neurotrophin delivery methods—their eventual  
48 depletion triggers an accelerated decline in neurite growth and survival of extant  
49 SGNs [13, 14, 15]. One major contributor is the structurally unstable nature of  
50 neurotrophins, which suffer from fragility and thermo-instability under normal  
51 physiological conditions both *in vitro* and *in vivo*, leading to short half-lives  
52 typically ranging from minutes to hours [16]. We set out to mitigate this phe-  
53 nomenon by utilizing the polyhedrin delivery system (PODS®)—a crystalline  
54 growth factor formulation developed to enable long-term release of growth fac-  
55 tors (e.g., neurotrophins) [17, 18, 19] (Figure 1B). The PODS® technology has  
56 adapted viral machinery to encase a chosen growth factor into polyhedrin pro-  
57 tein cases. The resultant growth factor co-crystals have slow degradation profiles  
58 under physiological conditions and, therefore, allow the sustained release of em-  
59 bedded bioactive growth factors.

60 We reasoned that a bio-engineered scaffolding incorporated with PODS®  
61 technology can establish a neuronal network between transplanted hPSC-derived

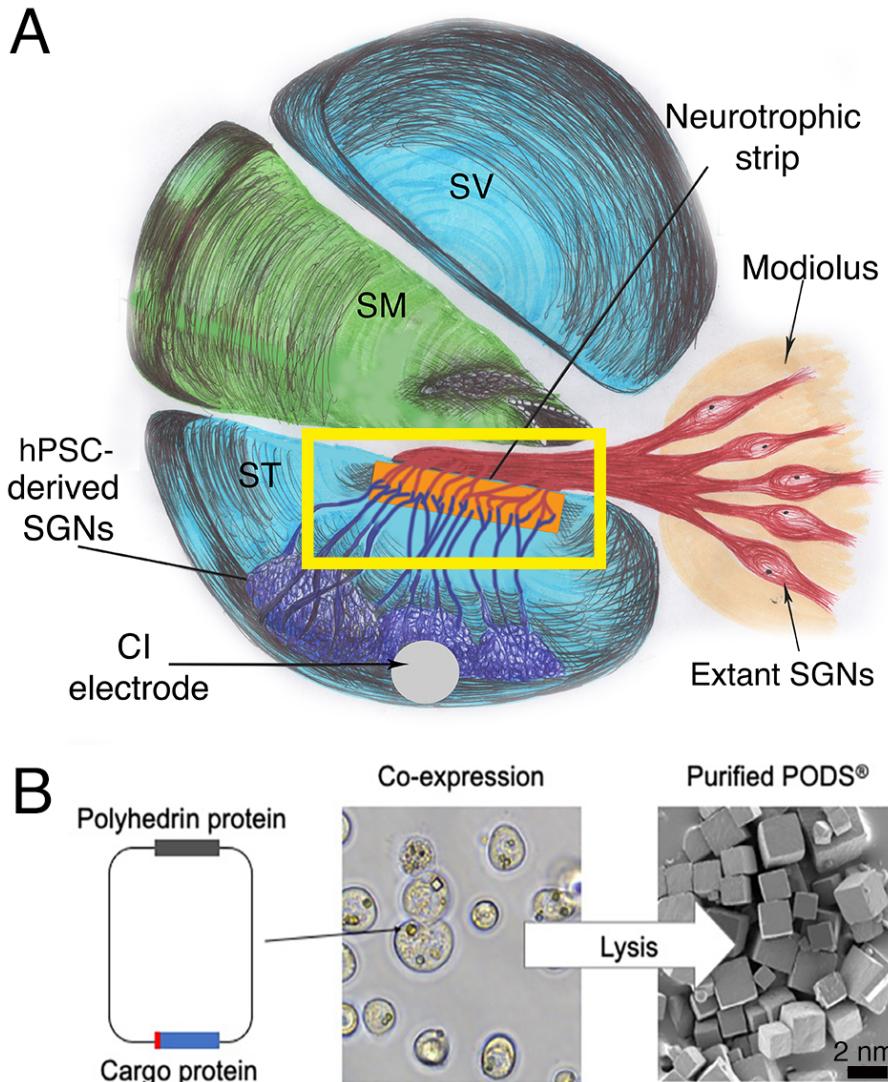


Figure 1: (A): A next-generation bioactive CI and a neurotrophic strip. The neural network in this scheme consists of a CI electrode, transplanted stem cell-derived SGNs, the neurotrophic strip, and extant/endogenous SGNs (red). Note that neuronal connection is largely absent with hair cells (not shown). (B): Polyhedrin Delivery System (PODS)®. PODS® crystals are cubic protein co-crystals produced by Sf9 cells (a clonal isolate of *Spodoptera frugiperda* Sf21 cells [IPLB-Sf21-AE], in which the polyhedrin protein and a second protein are co-expressed). This second protein is termed as the active, or cargo protein (Figure 1B, left). Inside the Sf9 cells, polyhedrin proteins self-assemble into cubic crystals (Figure 1B, center) while the active protein, tagged with a short peptide sequence, binds to the growing polyhedrin crystal. The crystals are extracted and purified by simple cell lysis and washes to remove cell debris (Scanning electron micrograph, Figure 1B, right).

62 ONP grafts and extant SGNs in the inner ear. More specifically, we hypothe-  
63 sized that PODS®-recombinant human neurotrophin system could stably pro-  
64 vide and maintain an adequate neurotrophin gradient to facilitate otic neuronal  
65 differentiation of and directional neurite outgrowth from hPSC-derived ONPs, .  
66 To test this hypothesis, we first devised a finite element model (FEM) to simu-  
67 late the *in vitro* neurotrophin gradient generated by PODS®. In this study, we  
68 focus on the role of BDNF—the most studied of the neurotrophins in the inner  
69 ear, and the most vital for the functional recovery of damaged SGNs [20]. For  
70 biological validation and demonstration we used a multi-chamber microfluidic  
71 device, that which mimics the *in vivo* micro-environment of the inner ear more  
72 so than conventional laboratory plates in terms of volume and concentrations  
73 of endogenous/exogenous factors [21].

74 **2. Materials and Methods**

75 *2.1. Polyhedrin delivery system*

76 The Polyhedrin Delivery System (PODS®-human BDNF [rhBDNF]) (Cell  
77 Guidance Systems, Cambridge, United Kingdom) was used as a self sustain-  
78 ing source of rhBDNF. PODS®-rhBDNF is composed of the polyhedrin pro-  
79 tein formed by *Bombyx mori*, an insect from the moth family *Bombycidae*. A  
80 cargo protein (i.e., rhBDNF) is co-expressed within the polyhedrin crystal and is  
81 slowly released by breakdown of the PODS® crystals via cell-secreted proteases  
82 (Figure 1B)[9, 18, 22].

83 *2.2. Human pluripotent stem cell culture using dual-compartment microfluidic  
84 device*

85 Human embryonic stem cells (hESCs: H7 and H9, passages number 25–35)  
86 and were obtained from WiCell Research Institute (Madison, Wisconsin, USA).  
87 Human induced pluripotent stem cells (hiPSCs: TC-1133HKK, passage num-  
88 ber 22–35) were generated from human CD34+ cord blood cells using the four  
89 Yamanaka factors at Lonza (Walkersville, Maryland [MD], USA). The hiPSC

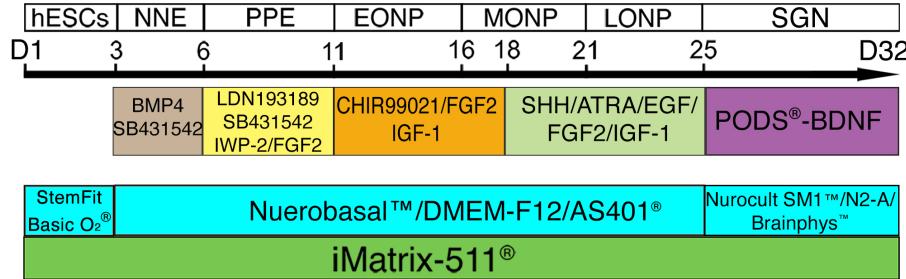


Figure 2: Overview of otic neuronal differentiation protocol for hPSCs. Please see detail in [8, 9, 23, 24] and the Supplemental Data. Each developmental stage is shown at the top, with key treatments shown below. NNE: nonneuronal ectoderm; PPE: preplacodal ectoderm; EONP: early-stage otic neuronal progenitor; MONP: mid-stage otic neuronal progenitor; LONP: late-stage otic neuronal progenitor; BMP4: bone morphogenetic protein 4; SHH: Sonic hedgehog; ATRA: all-trans retinoic acid; EGF: epidermal growth factor; IGF-1: insulin-like growth factor-1; FGF2: fibroblast growth factor 2; N2B27-CDM: chemically defined medium containing N2 and B27 supplements.

90 cell line (TC-1133HKK) was kindly provided by Healios K.K. (Tokyo, Japan).  
 91 hPSC-derived ONPs were derived based on our previously established protocol  
 92 (Supplemental Data) [8, 9, 23, 24]. A step-wise series of ligands and growth  
 93 factors was added to a neuronal induction medium to promote hPSC differen-  
 94 tiation toward the late-stage ONP lineage—mitotic progenitor population that  
 95 generates the SGNs. (Figure 2).

96 Microfluidic devices provide a platform for specifically evaluating axonal re-  
 97 generation [25]. Here, multi-chamber microfluidic devices, Xona™ Microfluidics  
 98 XC150 and XC450 (Xona™ Microfluidics, Research Triangle Park, North Car-  
 99 olina, USA), were used for computational calculation and biological validation  
 100 (Figure 3A–B) of an FEA. The Xona™ device allows for neurites to grow to-  
 101 ward growth factors in the opposite chamber while limiting migration of derived  
 102 ONP cell bodies due to specific dimensions of the device. Additionally, the mi-  
 103 crochannel array between the two chambers mimics the porous bony separation  
 104 (osseous spiral lamina) between the modiolus (where extant SGNs are localized)  
 105 and the scala tympani (where the biohybrid CI will be implanted). Thus the

<sup>106</sup> diffusion profile of the released rhBDNF *in vitro* more accurately predicts that  
<sup>107</sup> of the *in vivo*.

<sup>108</sup> The devices were washed and coated with poly-L-ornithine (PLO, 20 µg/mL  
<sup>109</sup> in H<sub>2</sub>O, Sigma-Aldrich, St. Louis, Missouri [MO], USA) and recombinant  
<sup>110</sup> laminin-511 (iMatrix-511, 0.5 mg/mL, Nacalai USA, San Diego, California [CA],  
<sup>111</sup> USA) according to the manufacturer-outlined protocol. Next, approximately  
<sup>112</sup> 1.75 x 10<sup>5</sup> cells (in 20 µL of media) were added through the top and bottom left  
<sup>113</sup> wells into the somal compartment (i.e., total amount of 3.5 x 10<sup>5</sup> hPSC-derived  
<sup>114</sup> ONPs were added).

<sup>115</sup> PODS®-rhBDNF were placed in the top right well of the neurotrophin com-  
<sup>116</sup> partment (Figure 3A–B) to generate a rhBDNF concentration gradient to pro-  
<sup>117</sup> mote directional neurite growth. hPSC-derived ONPs were cultured for 7 days  
<sup>118</sup> in the Xona™ device to induce otic neuronal differentiation. Note that high-  
<sup>119</sup> density cell cultures were induced to facilitate molecular studies as well as the  
<sup>120</sup> generation of a more biologically relevant neuronal phenotype (i.e., otic lineage)  
<sup>121</sup> [25]. Media was topped off daily after imaging (from 20-40 µL per well).

<sup>122</sup> 2.3. *Enzyme-linked immunosorbent assay for brain-derived neurotrophic factor*

<sup>123</sup> In order to determine the breakdown and release kinetics of PODS®-rhBDNF,  
<sup>124</sup> an experiment measuring rhBDNF concentrations at sequential time points was  
<sup>125</sup> performed. The culture media from both a control and experimental condition  
<sup>126</sup> were collected at each time point and immediately stored at -80°C before run-  
<sup>127</sup> ning an enzyme-linked immunosorbent assay (ELISA) after the final collection.  
<sup>128</sup> The same method was applied to measure the degradation kinetics of rhBDNF  
<sup>129</sup> protein with a carrier protein (Bovine Serum Albumin [BSA]) (#248-BDB-050,  
<sup>130</sup> R&D Systems, Minneapolis, Minnesota, USA). Experimental conditions were  
<sup>131</sup> culture media enriched with 10% fetal bovine serum (FBS) (Thermo Fisher  
<sup>132</sup> Scientific, Waltham, MA, USA). All rhBDNF samples were quantified with a  
<sup>133</sup> BDNF ELISA kit (# BGK23560; PeproTech, Rocky Hill, New Jersey, USA),  
<sup>134</sup> and the results were analyzed with a Synergy HTX Multi-Mode Reader (BioTek,  
<sup>135</sup> Winocski, Vermont, USA) at a 450 nm wavelength, as instructed by the man-

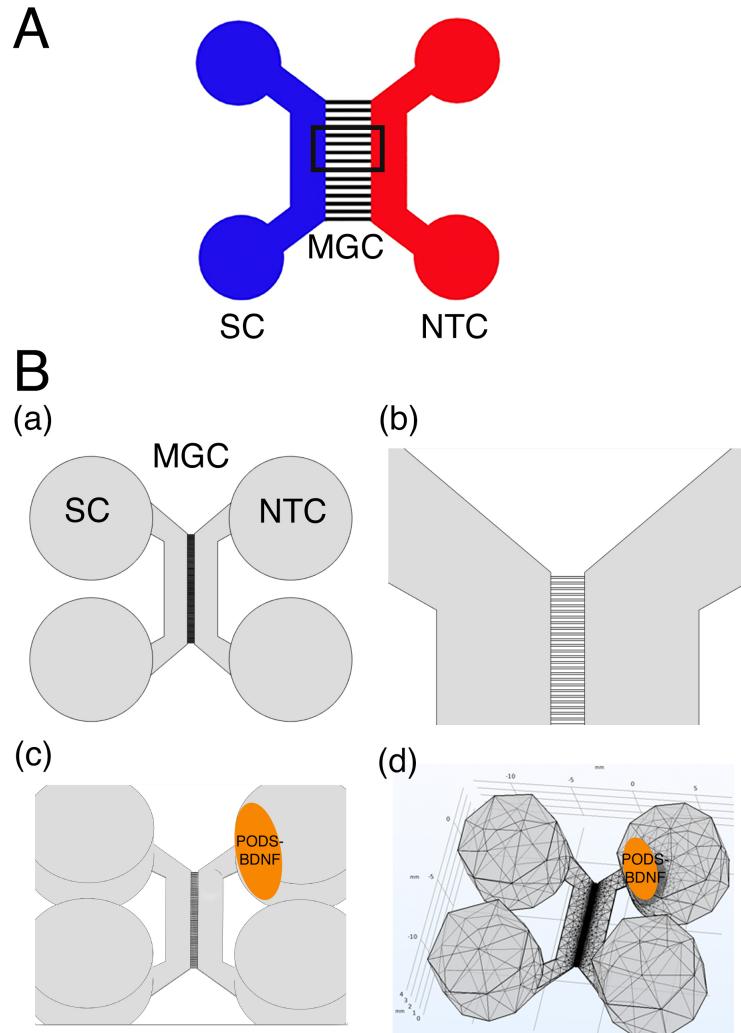


Figure 3: (A): Schematic specification of a Xona™ Microfluidics XC 450. Two main channels (somal compartment (SC) and neurotrophin compartment (NTC)) on either side are connected by a micro-groove channel (MGC) spanning 450  $\mu\text{m}$  with a width of 10  $\mu\text{m}$  (black square). The somal compartment contains hPSC-derived late-stage ONPs (shown in black), while the neurotrophin compartment contains the secreted factors (i.e., BDNF) that are released from PODS®-rhBDNF crystals. (B):(a) Xona™ Microfluidics XC450 device geometry, created at a 1:1 scale with Autodesk Inventor®, in which the diffusion profile of the released BDNF was modeled. (b) Detail of the microchannels adjoining the two compartments of the Xona™ Microfluidics XC450. (c) Xona™ Microfluidics XC450 device showing the optimal area and geometry to localize PODS®-rhBDNF (yellow ellipsoid area). (d): Mesh geometry of the Xona™ XC450 generated with Autodesk Inventor, depicting the localized volume of PODS®-rhBDNF (1  $\mu\text{L}$ ) as an ellipsoid disk.

<sup>136</sup> ufacturer. Molecular kinetics were then calculated using the MATLAB Curve  
<sup>137</sup> Fitting Toolbox (MathWorks, Natick, CA, USA).

<sup>138</sup> *2.4. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis*

<sup>139</sup> Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is  
<sup>140</sup> commonly used as a method to separate proteins with molecular masses between  
<sup>141</sup> 5 and 250 kDa [26], a range of which is suitable for detecting recombinant hu-  
<sup>142</sup> man BDNF (molecular weight [MW]: 14kDa [27]) and polyhedrin (MW: ~29  
<sup>143</sup> kDa [22]). In this study, SDS-PAGE was used to assess the molar ratio of poly-  
<sup>144</sup> hedrin to BDNF at different quantities. Briefly, each protein sample was diluted  
<sup>145</sup> in deionized water and mixed with 6x Laemmli sample buffer (Bio-Rad Labora-  
<sup>146</sup> tories, Inc., Des Plaines, Illinois [IL], USA) containing 2-mercaptoethanol and  
<sup>147</sup> heated at 100°C for 5 to 20 minutes. Samples were then loaded into precast  
<sup>148</sup> Mini-PROTEAN TGX 4-15% polyacrylamide mini-gels (Bio-Rad Laboratories,  
<sup>149</sup> Inc., Des Plaines, IL, USA). Then, 5 mL of Precision Plus Protein Kaleido-  
<sup>150</sup> scope Prestained Protein Standards (Bio-Rad Laboratories, Inc., Des Plaines,  
<sup>151</sup> IL, USA) were loaded in each gel run. Electrophoresis was performed at room  
<sup>152</sup> temperature for approximately 90 minutes using a constant voltage (100V) in  
<sup>153</sup> 1x solution of Tris-Glycine-SDS electrophoresis buffer (Bio-Rad Laboratories,  
<sup>154</sup> Inc., Des Plaines, IL, USA) until the dye front reached the end of the 60 mm  
<sup>155</sup> gel. After electrophoresis, the mini-gels were rinsed with deionized water 3  
<sup>156</sup> times for 5 minutes and were subsequently incubated in SimplyBlue™ SafeStain  
<sup>157</sup> (ThermoFisher Scientific, Waltham, MA, USA) for one hour at room temper-  
<sup>158</sup> ature with gently agitation. Images obtained from gels were analyzed using  
<sup>159</sup> ImageJ 1.53g (December 4, 2020, the National Institutes of Health, Bethesda,  
<sup>160</sup> MD, USA [28]). The calculated molar ratio was applied to the COMSOL®  
<sup>161</sup> Multiphysics model to accurately predict the amount of rhBDNF released from  
<sup>162</sup> PODS®-rhBDNF. SDS-PAGE was performed according to the manufacturer's  
<sup>163</sup> technical guide (Bio-Rad Laboratories, Inc., Des Plaines, IL, USA).

164     *2.5. Western Blot*

165     The identity of the rhBDNF protein detected by SDS-PAGE was verified  
166     by western blot (Bio-Rad Laboratories, Inc., Des Plaines, IL, USA). Briefly,  
167     the polyvinylidene difluoride (PVDF) membrane was prepared in methanol for  
168     30 seconds before soaking in 1x Tris-Glycine-Methanol transfer buffer for 10  
169     minutes. Wet transfer was performed at 4°C for approximately 60 minutes us-  
170     ing a constant voltage (100V) in 1X solution of Tris-Glycine-Methanol transfer  
171     buffer. After transfer, the membrane was briefly rinsed with 1X Tris-buffered  
172     saline Tween-20 (TBST) and was subsequently blocked in 5% BSA in TBST for  
173     24 hours at 4°C with gentle agitation. The membrane was then rinsed with 1x  
174     TBST before incubating in BDNF polyclonal antibody (ThermoFisher Scien-  
175     tific, Waltham, MA, USA) diluted to 1:1500 in 1% BSA in TBST for 24 hours at  
176     4°C with gentle agitation. Following incubation, the membrane was rinsed in 1x  
177     TBST 5 times for 5 minutes to remove unbound primary antibody. Then, the  
178     membrane was incubated in Goat anti-Rabbit IgG (H+L) horseradish peroxy-  
179     dase (HRP) conjugated secondary antibody (ThermoFisher Scientific, Waltham,  
180     MA, USA) diluted to 1:5000 in 1% BSA in TBST for one hour at room tem-  
181     perature with gentle agitation. Following incubation, the membrane was rinsed  
182     in 1x TBST 5 times for 5 minutes to remove unbound secondary antibody. For  
183     sensitive detection, the membrane was treated with Pierce™ ECL Western Blot-  
184     ting Substrate (ThermoFisher Scientific, Waltham, MA, USA), and visualized  
185     using an Azure 600 Digital Imager (Azure Biosystems, Dublin, CA, USA). Elec-  
186     trophoresis buffer for sample condition and run condition was summarized in  
187     Supplementary Table S1.

188     *2.6. Three-dimensional finite element analysis*

189     We used finite element analysis (FEA) to simulate the BDNF concentration  
190     gradient over time in a multi-chamber microfluidic device. FEA is a compu-  
191     tational numerical technique, which approximates mathematical solutions to  
192     partial differential equations (PDEs) that appropriately simulate complex real-  
193     world problems including stress/strain testing, thermal conduction, and diffu-

194 sion in various geometries and materials. In this study, the FEM allowed us to  
195 predict the concentration gradient with respect to time depending on the num-  
196 ber of PODS®-rhBDNF introduced into the system. To solve the FEM, we used  
197 COMSOL® Multiphysics (version 5.6 [released on November 11, 2020], COM-  
198 SOL, Inc., Burlington, Massachusetts [MA], USA), which is a finite element  
199 method solution tool for engineering and scientific research computations. We  
200 used sustained-release kinetics for PODS®-rhBDNF determined from aforemen-  
201 tioned ELISA studies, SDS-PAGE, as well as data from a previous study from  
202 our group [9]. Device geometry was generated at a 1:1 scale using Autodesk®  
203 Inventor 2019.0.2 (Autodesk, Mill Valley, CA, USA) (Figure 3B). The com-  
204 putational analysis was implemented on a high-performance desktop computer  
205 platform equipped with a 64 GB RAM CPU (AMD Ryzen Threadripper 3990X  
206 64-Core, 128-Thread @ 4.3 GHz) and two GPU cards (NVIDIA GeForce RTX  
207 3080Ti, 12GB 384-bit GFF6X Graphics card).

208 *2.7. Immunocytochemistry and image acquisition*

209 Microfluidic devices were coated with poly-d-lysine (PDL) (#A3890401,  
210 ThermoFisher Scientific, Waltham, MA, USA) and poly-ornithine (PLO) (#  
211 A-004-C, MilliporeSigma, St. Louis, MO, USA) as per the manufacturer's in-  
212 structions. A total of 100,000 dissociated hPSC-derived ONPs were plated into  
213 the somal compartment of the device. On Day 7, 4% (w/v) paraformaldehyde  
214 (PFA) (ThermoFisher Scientific, Waltham, MA, USA) was added to the com-  
215 partments for 20 minutes to fix the cells. ICC was used to stain for GATA3,  
216 PAX8, and beta-III tubulin. These three proteins have shown to appropriately  
217 characterize ONPs in our previous studies [8, 9, 24]. Following PBS wash, cul-  
218 tures were blocked with 5% BSA at room temperature for 1 hour. Cultures  
219 were then incubated overnight at 4°C on a shaker plate in primary antibody  
220 solution using Rabbit anti-Beta-III-Tubulin (1:100, Abcam, Cambridge, MA,  
221 USA), Goat anti-PAX8 (1:500, Abcam, Cambridge, MA, USA), and Mouse  
222 anti-GATA3 (1:500, R&D Systems, Minneapolis, MN, USA). Following PBS  
223 washes, cultures were incubated at room temperature for 90 minutes on a

shaker plate in secondary antibody solution composed of Alexafluor647 anti-Rabbit (1:1000, ThermoFisher Scientific, St. Louis, MO, USA), Alexafluor488 anti-Goat (1:1000, ThermoFisher Scientific, St. Louis, MO, USA), and Alexafluor594 anti-Mouse (1:1000, ThermoFisher Scientific, St. Louis, MO, USA) in 1% BSA. Following PBS -/- washes, cultures were incubated with DAPI for 20 minutes (300 nM, ThermoFisher Scientific, St. Louis, MO, USA). Secondary antibody controls were performed each time multiple primary antibodies were used [29]. Labeling controls (detection controls) were performed for a sample from each batch of hPSC culture [29]. See Supplementary Figure S1 for exemplary figures for these control conditions. Results were imaged using a Nikon Ti2 Widefield Laser Microscope System (Nikon, Tokyo, Japan). Phase-contrast images were captured on a Nikon Eclipse TE2000-U inverted microscope (Nikon, Tokyo, Japan). All fluorescence images were acquired on a Zeiss LSM 510 META laser-scanning confocal microscope (Carl Zeiss, Oberkochen, Germany), a Nikon Ti2 laser scanning confocal microscope (Nikon, Tokyo, Japan), or a Leica SP5 laser-scanning confocal microscope (Leica, Welzlar, Germany). Observers were blinded to the conditions during imaging and tracing. In general, the images were processed with a Image J ver. 1.53j or Matlab R2020b. Further detail on image acquisition and quantification of fluorescent-positive cells can be found in the Supplemental Data.

#### 2.8. Preferred cell orientation analysis

Collective cell migration, where cells organized in a tightly connected fashion migrate as cohesive structures, is a critical biological process to highlight the neurotrophin diffusion profile [30]. To evaluate this process, time-lapse acquisition of images of the Xona™ device was performed using an inverted microscope (Nikon Eclipse TS100, Tokyo, Japan) at Day 1, 3, 5, and 7. Due to the high cell density required for hPSC-ONPs to survive in the somal compartment of the Xona™ device, images were not amenable to manual analysis in most of the cases. To circumvent this problem, we performed a series of image pre-processings that are mainly based on modified binarization-based extraction of

254 alignment score methods with some modifications [31]. We used MATLAB Im-  
255 age Processing Toolbox R2020b (version 9.9.0.1495850, September 30th, 2020,  
256 Mathworks, Natick, MA) for analysis. Please see Supplementary Figure S2 for  
257 further detail. The analysis of directional data in general represents a particular  
258 challenge: there is no reason to designate any particular point on the circle as  
259 zero, and it is somewhat arbitrary depending on where one sets a coordinate  
260 [32, 33]. In this study, we used polar coordinates to determine the directionality  
261 of preferred cell orientation. For this analysis, we again used MATLAB Image  
262 Processing Toolbox R2020b. See detailed discussion on how we determined the  
263 preferred cell orientation in Supplementary Figure S3.

264 *2.9. Neurite alignment vector assay, neurite growth assay and cell migration*  
265 *assay*

266 The microfluidic device allowed us to culture hPSC-derived ONPs in a po-  
267 larized manner and to directly isolate/analyze neurites. To evaluate the neurite  
268 projection into the neurotrophin compartment by derived otic neurons cultured  
269 in the somal compartment, we performed a neurite alignment vector assay. We  
270 also evaluated the length of neurites that grew from hPSC-derived ONPs. For  
271 these purposes, hPSC-derived ONPs were cultured in a Xona™ XC450 for seven  
272 days and then immunostained with *beta*-III tubulin and DAPI. We used two  
273 ImageJ plug-in toolkits, NeuriteTracer and NeuronJ, to assess neurite align-  
274 ment and neurite growth [34, 35]. Due to the bipolar nature of hPSC-derived  
275 ONPs/SGNs, we measured the two longest neurites from the cells [24, 36].  
276 Please see Supplementary Figure S4 for detailed description of this analysis. We  
277 used hPSC-derived ONPs cultured with 800,000 PODS®-rhBDNF as a positive  
278 control. The quantity 800,000 was chosen based on our FEM in that there was  
279 no neurotrophin gradient in the somal compartment. As a negative control, we  
280 used 20 ng/mL of recombinant human BDNF. To evaluate cell migration across  
281 the microgroove channels, we performed cell migration analysis. We manually  
282 counted the number of ONPs that migrated from the somal compartment into  
283 the microchannels and neurotrophin compartment.

284 *2.10. Statistical analysis*

285 When appropriate, and as indicated in each figure, statistical analysis was  
286 performed. Experimental values are typically expressed as mean and standard  
287 error (SE). The majority of the statistical analyses were performed with Python  
288 3.9.6. (Python Software Foundation, Wilmington, Delaware, USA). The follow-  
289 ing libraries were used for the statistical analysis: Scipy, Numpy, Matplotlib,  
290 and Seaborn [37, 38, 39]. Normal distributions were assumed unless mentioned  
291 otherwise. *P* values smaller than 0.05 were considered statistically significant.  
292 For circular statistics, we derived the sample mean vector and its polar coor-  
293 dinate. Mean and confidence intervals were calculated. We chose confidence  
294 coefficient, *Q*, e.g. *Q* = 0.95. To analyze the axial nature of data, especially to  
295 compute the mean vector angle, we doubled each angle and reduced the mul-  
296 tiples modulo 360°. Please see detailed discussion in Supplementary Figure S3  
297 and S5. The Rayleigh test of uniformity and V-test were performed to deter-  
298 mine whether the samples differ significantly from randomness (i.e., where there  
299 is statistical evidence of directionality). One-sample test for the mean angle was  
300 performed to test whether the population mean angle is statistically different  
301 from the given angle. In all of our circular statistics, von Mises distribution was  
302 assumed and also verified. Circular statistics were performed using CircStat: A  
303 MATLAB Toolbox [33, 40]. Please see detailed description of circular statistics  
304 in Supplementary Figure S3 and S5. Experiments were done in three biological  
305 replicates unless otherwise specified in Figure captions.

306 **3. Results**

The appropriate number of PODS®-rhBDNF crystals to induce an effective neurotrophin gradient for otic neuronal differentiation and directed neurite outgrowth was calculated using a three-dimensional FEA that predicts the concentration profile of BDNF formed through the gradual release and diffusion of BDNF from PODS®-rhBDNF. First, we quantified the chemical kinetics of this phenomenon with ELISA testing (Figure 4) to establish the parameters for

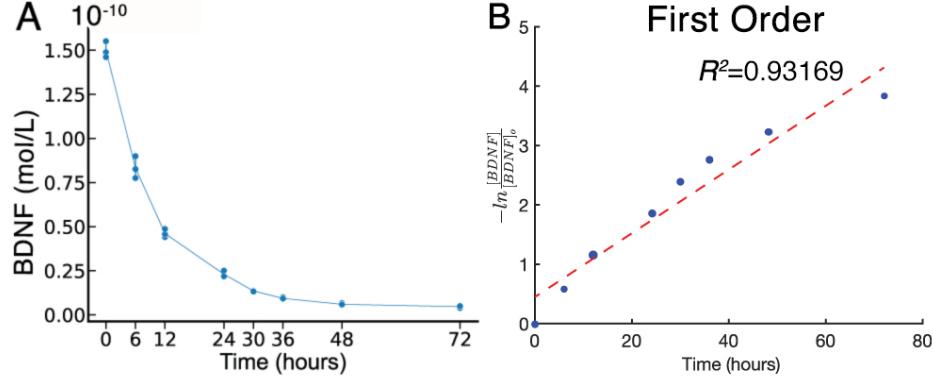
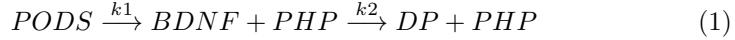


Figure 4: Chemical kinetics of Brain Derived Neurotrophic Factor (BDNF). (A): Concentration of rhBDNF in solution over 72 hours after initial application. (B):  $\frac{1}{[BDNF]}$  data fit to first order curve. Please see other curve fit data in Supplemental Figure 1. Red line: fitted line, blue dots: individual data point.  $k_2$  is defined as slope of fitted curve.

the FEA. Here, two consecutive chemical reactions occur: 1) the breakdown of PODS® crystals into polyhedrin protein and rhBDNF, and 2) the degradation of rhBDNF toward the degradation product (Equation (1)).



where  $DP$  is the degradation product of the released rhBDNF, PHP is the polyhedrin protein, and  $k_1$  and  $k_2$  are the rate constants ( $\frac{1}{hour}$ ) for their respective reactions.

Degradation kinetics data for rhBDNF was collected while monitoring rhBDNF concentration after introducing a predefined amount into a single well of solution. The data obtained throughout the first 72 hours indicate an exponential decay, suggesting first order kinetics (Figure 4). To confirm this notion, we performed a linear and nonlinear least square analysis of the kinetic data with the MATLAB Curve Fitting Toolbox. We found that the corresponding  $R^2$  was 0.93169 for the first order curve-fit, confirming that the degradation kinetics was indeed first order. The rate constant for a first order reaction is defined to be slope of the time plot of the logarithmic ratio between concentration and

319 initial concentration. The value for  $k_2$  ( $0.0679 (\frac{1}{hour})$ ) is the slope of the loga-  
320 rithm of the ratio between concentration and initial concentration (See further  
321 detail in Supplementary Figure S7). Furthermore, data for the complete chemi-  
322 cal reaction were collected by similarly monitoring rhBDNF concentration over  
323 time after placing a predefined amount of PODS®-rhBDNF into a single well  
324 of solution. The data collected appeared to fit the curve for Equation 2, which  
325 describes the concentration of the intermediate product of two consecutive first  
326 order reactions:

$$C_{rhBDNF} = C_{PODS} \cdot k_1 \cdot \left( \frac{e^{-k_1 \cdot t}}{k_2 - k_1} + \frac{e^{-k_2 \cdot t}}{k_1 - k_2} \right) \quad (2)$$

327 where  $C_{rhBDNF}$  is the concentration of rhBDNF and  $C_{PODS}$  is the concentra-  
328 tion of PODS® [43].

329 We successfully fit the data to this equation's respective curve and empiri-  
330 cally approximated  $k_1$  to be  $0.00686 (\frac{1}{hour})$  after plugging in our value for  $k_2$   
331 (See further detail in Supplementary Figure S7).

332 SDS-PAGE was used to separate PODS®-rhBDNF crystals into its con-  
333 stituent proteins to determine the molar ratio of polyhedrin to rhBDNF. Vi-  
334 sualization with Coomassie G-250 solution (Figure 6A) revealed three distinct  
335 protein bands at 18.8 (a), 28.0 (b), and 46.8 (c) kDa, which correspond with  
336 the molecular weights of H1-tagged BDNF monomer, polyhedrin, and H1-tagged  
337 BDNF monomer attached with polyhedrin, respectively. Western blot analy-  
338 sis was subsequently conducted to confirm the identity of the 18.8 kDa band  
339 as rhBDNF. Enhanced chemiluminescence (Figure 6B) revealed four protein  
340 bands at 14.0 (a), 18.8 (b), 37.6 (c), and 46.8 (d) kDa, which correspond with  
341 the molecular weights of rhBDNF monomer, H1-tagged BDNF monomer, H1-  
342 tagged BDNF dimer, and H1-tagged BDNF monomer attached with polyhedrin.  
343 Immunoblot detection of the 18.8 kDa band further implicates its identity as  
344 rhBDNF. SDS-PAGE images were converted to 8-bit grayscale and mean cor-  
345 rected integrated pixel intensity values were calculated for protein bands located  
346 at 28.0 and 18.8 kDa; the protein band detected at 46.8 kDa was omitted from

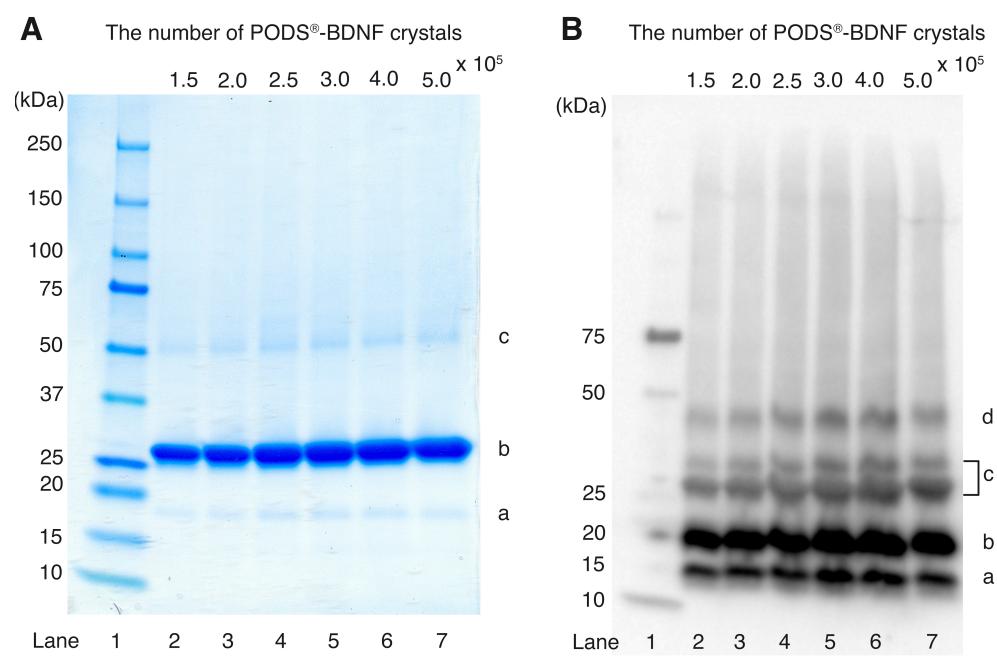
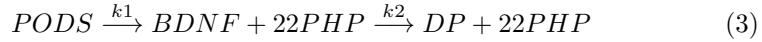


Figure 5: (A): SDS-PAGE analysis of PODS®-rhBDNF. Samples containing six quantities of PODS®-rhBDNF crystals were run on precast 4-15% polyacrylamide mini-gels and stained with Coomassie G-250 solution for visualization. Protein bands appear at approximately 18.8 (a), 28.0 (b), and 46.8 kDa (c). Lane 1: molecular weight marker. Lanes 2-7: samples containing

<sup>347</sup> the final computation based on the fact that it contained a 1:1 ratio of poly-  
<sup>348</sup> hedrin to BDNF. Results indicate that the molecular ratio of polyhedrin to  
<sup>349</sup> rhBDNF is approximately 22:1. This transforms Equation (1) into:



<sup>350</sup> Using these calculated rate constants with the calculated molar ratio, the  
<sup>351</sup> resulting chemical gradient over time after PODS®-rhBDNF placement can be  
<sup>352</sup> solved for any geometry by applying Fick's second Law of diffusion (Equation  
<sup>353</sup> 4) and the appropriate boundary (Equations 5 and 6) and initial conditions  
<sup>354</sup> (Equation 7):

$$\frac{dC}{dt} = \nabla \cdot (\nabla C \cdot D) - k_2 \cdot C \quad (4)$$

<sup>355</sup> Boundary Conditions:

$$\delta C \Big|_{walls} = 0 \quad (5)$$

<sup>356</sup> and

$$\delta C \Big|_{PODS} = k_1 \cdot PODS_0 \cdot e^{-k_1 \cdot t} \quad (6)$$

Initial conditions:

$$C \Big|_{t=0} = 0 \quad (7)$$

<sup>357</sup> where  $C$  is the concentration of rhBDNF,  $D$  is diffusivity of rhBDNF (6.76  
<sup>358</sup>  $\frac{mm^2}{day}$  [44]),  $-k_2 \cdot C$  is the sink term corresponding to the degradation and cell-  
<sup>359</sup> utilization of the rhBDNF, and  $PODS_0$  is the initial concentration of the cargo  
<sup>360</sup> protein (i.e., BDNF) within the PODS® crystals. The first boundary condition  
<sup>361</sup> (Equation 4) shows that the concentrations of rhBDNF at the walls of the  
<sup>362</sup> microfluidic device are fixed at 0. The second boundary condition (Equation 5)  
<sup>363</sup> represents the exponential nature of the decay of PODS®. Note that both are  
<sup>364</sup> Neumann boundary conditions.

365 We empirically tested two available microchannel lengths—(i.e., Xona<sup>TM</sup>-  
366 XC150 [150  $\mu\text{m}$ ] and Xona<sup>TM</sup>-XC450 [450  $\mu\text{m}$ ]). This was done first because  
367 mass (i.e., BDNF) transport from the neurotrophin compartment through the  
368 micro-groove channels into the somal compartment is an important factor in  
369 generating the concentration gradient *via* diffusion mixing. We determined that  
370 the Xona<sup>TM</sup> Microfluidics XC450 was more appropriate for this study as the XC-  
371 150's micro-groove channels were not long enough to generate the appropriate  
372 concentration gradient throughout the somal compartment. This feature is rel-  
373 evant to human inner ear because the micro-groove channels in the Xona device  
374 simulates the presence of the osseous spiral lamina and modiolus between the  
375 scala tympani and SGNs [41, 42]. Following device selection, we generated a  
376 three-dimensional geometry mesh of the XC450 for the FEA (Figure 3B(d)).  
377 Please see Supplementary Figure S6 for detailed measurements of the mesh.

378 The finite element model was then computed for different PODS<sup>®</sup>-rhBDNF  
379 concentrations and time intervals to empirically optimize the rhBDNF concen-  
380 tration gradient for hPSC-derived ONP differentiation into SGNs as well as  
381 directed neurite extension. Figure 6 shows FEM-computed rhBDNF concen-  
382 tration gradients for 20,000 PODS<sup>®</sup>-rhBDNF from Day 1–7. Note that the  
383 rhBDNF concentrations were greater on D2–5 to promote neuronal differentia-  
384 tion and neurite outgrowth observed on D7 (Figure 6B). Computed diffusion flux  
385 was uniform throughout D1–7 (Figure 6C). Also note that highest concentration  
386 of rhBDNF released from PODS<sup>®</sup>-crystals was greater than 50 pg/mL, the con-  
387 centration sufficient for otic neuronal differentiation and neurite outgrowth of  
388 hPSC-derived ONP 3D spheroids determined in our previously published data  
389 [9]. Optimization of the adequate number of PODS<sup>®</sup>-rhBDNF was performed  
390 empirically; we also performed FEA with 10,000 and 40,000 PODS<sup>®</sup>-rhBDNF.  
391 Please see detailed discussion for the empirical optimization in Supplementary  
392 Figure S8.

393 To objectively compare the degree of otic neuronal differentiation in hPSC-  
394 derived ONPs, we performed quantitative analysis of PAX8 and GATA3 double-  
395 positive cells using immunocytochemistry. We chose PAX8 and GATA3 for this

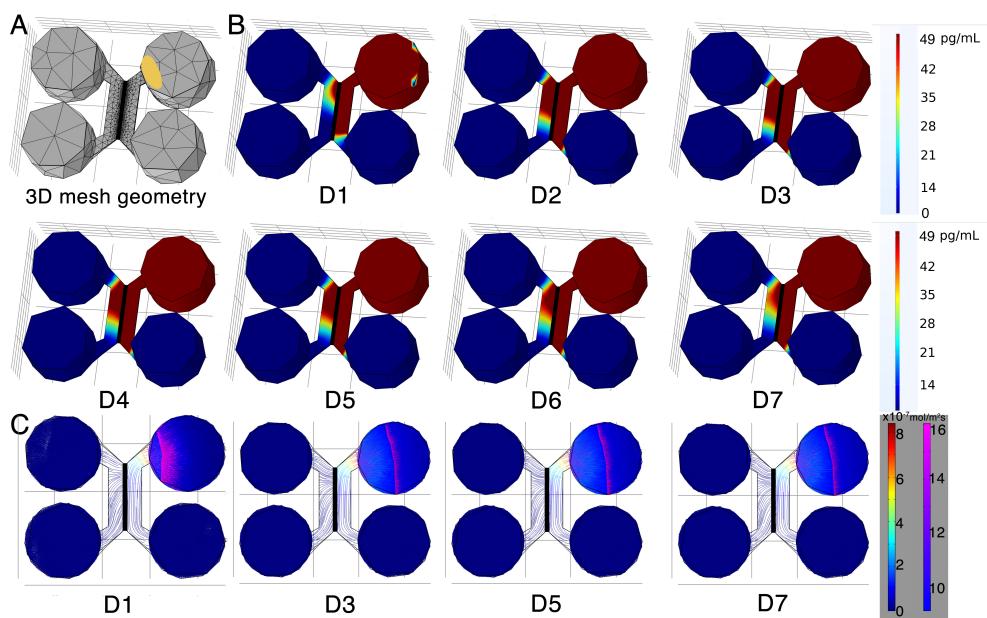


Figure 6: (A): 3D mesh geometry of a Xona™ XC450 created with Autodesk Inventor. A PODS®-rhBDNF ellipsoid disc is shown in yellow. (B): rhBDNF concentration gradient for 20,000 PODS®-rhBDNF from D1–D7. Note that the model does not account for media changes during the culture period. The corresponding color map shown has a range from 0 ng/mL to 49 pg/mL. (C): Diffusion flux ( $\text{mol}/\text{m}^2\text{s}$ ) was computed using COMSOL Chemical Engineering module on D1, D3, D5, and D7.

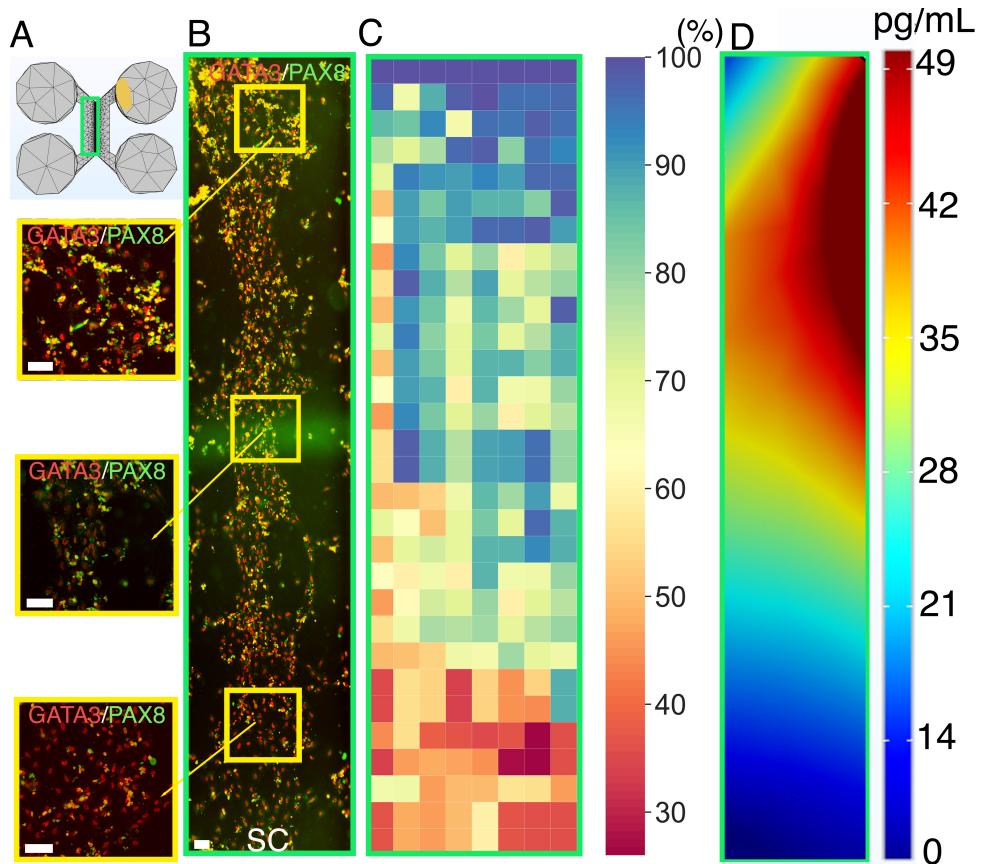


Figure 7: Human PSC-derived late-stage ONPs grown in a microfluidic device at Day 7 were stained for GATA3 (red) and PAX8 (green). (A): Geometry mesh of the microfluidic device Xona™ XC450 generated by Autodesk Inventor. The green square indicates the cell seeding location in the somal compartment of the device. An ellipse-shaped PODS®-rhBDNF disc is shown in yellow. (B): Cells were analyzed by staining with neuronal markers GATA3 (red) and PAX8 (green). Yellow highlighted regions are magnified on the left. SC: somal compartment. (C): Heat-map representation of double-positivity of hPSC-derived ONPs in the device for GATA3 and PAX8 ( $n = 3$ ). Double-positivity increases from the lower to the upper portion of the somal compartment, suggesting that the higher BDNF concentration in the upper portion of the device improves differentiation. Scale bar: 100  $\mu\text{m}$ . (D): Prediction of BDNF concentration gradient released from PODS®-rhBDNF at seven days in culture using a finite element model. The corresponding color map is shown with a range from 0–49 pg/mL.

analysis because our previous studies indicated high expression of these protein markers in late-stage ONPs and early-stage SGNs [8, 9, 24]. Cells were stained in the somal compartment of the Xona™ device, highlighted in green in Figure 7A. Figure 7B shows the resulting image of cells in the somal compartment, and a heat-map representation of the percentage of double-positive cells is shown in Figure 7C. It should be noted here that the heatmap is sensitive to the differences in cell density across channel. This was accounted by averaging the double-positivity across three biological replicates. The heat-map indicates higher double-positivity in the upper region of the somal compartment, which is closest to the PODS®-rhBDNF disc placement (shown in a orange ellipse in Figure 7A) in the neurotrophin compartment. Double-positivity decreases in the somal compartment as distance from the PODS®-rhBDNF disk increases, supporting the presence of a BDNF neurotrophin gradient as predicted by our computational model calculation (Figure 7D).

We defined two hypothetical directional angles to predict the orientation of hPSC-derived ONPs and neurite growth (Figure 8). The  $n$ -dimensional Euclidean space, denoted by  $\mathbb{R}^n$ , is a linear vector space in that we can use polar coordinates to compute the directionality of cells and neurites [45]. Here, we used  $n = 1$  and  $2$ . For one-dimensional Euclidean space ( $n = 1$ ), we simply drew a line for the Euclidean distance—the shortest distance between two points as shown in Figure 8A(b) (dark green lines). The two points were 1) the center point of a PODS®-rhBDNF disk ( $P$ ) and 2) the mid point of the medial side ( $Q_{1-5}$ ) (i.e., the near side to microgroove channels) of a pre-designated square (shown as a black square, zone 1–5 in Figure 8), respectively. The Euclidean distance angle (EDA),  $\phi_i$ , was defined as the angle between the horizontal line zero direction and the line  $PQ_i$  that consists of the Euclidean distance where  $i = 1 - 5$ .

For two-dimensional Euclidean space ( $n = 2$ ), we utilized Fick's first law, which dictates that the diffusion flux ( $D$ ) is proportional to the concentration gradient ( $C$ ) [46]. Based on this theorem, the direction of a flow vector can be used to represent concentration gradient for the directionality. We hypoth-

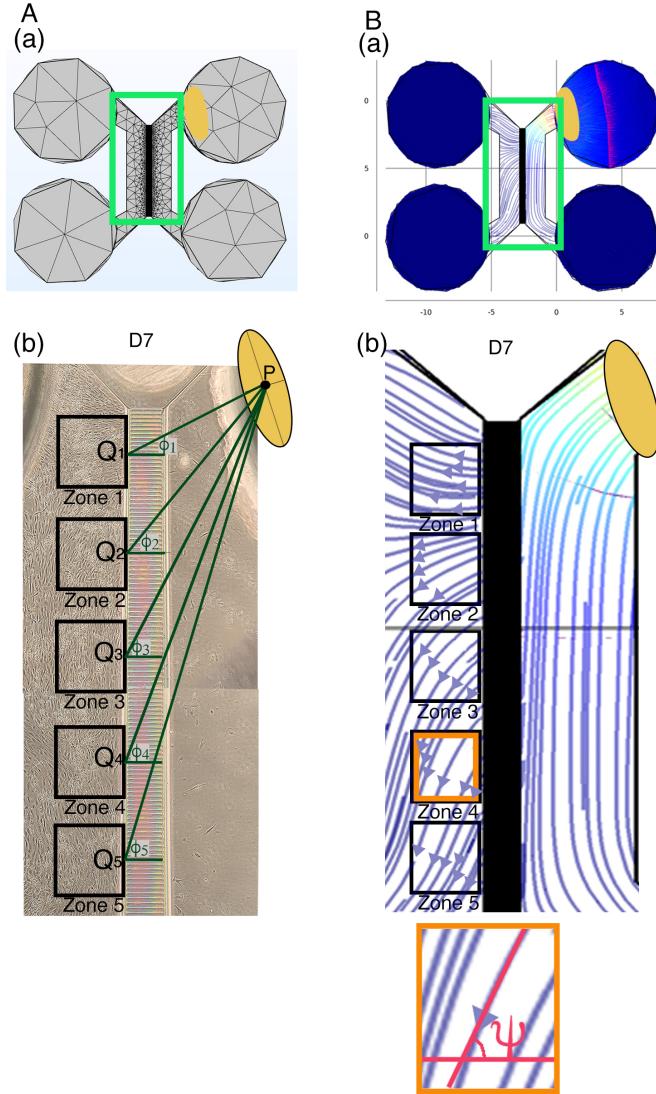


Figure 8: (A): Defining the Euclidean distance angle (EDA). (a) X-Y plane geometry mesh of a Xona™ XC450 device. Green square shows an area corresponding to a phase-contrast image below. An ellipse-shaped PODS®-rhBDNF disc is shown in yellow. (b) hPSC-derived ONPs were cultured in a Xona™ XC450 for seven days (D7). Yellow ellipse once again indicates the location of a disk contains PODS®-rhBDNF crystals (P: the center of this ellipse). In the somal compartment of the Xona™ XC450 device, we defined Zone 1–5, shown in black square. A line was drawn from the center of the PODS®-rhBDNF disk (P) to (Q<sub>1–5</sub>) (i.e., the near side to microgroove channels) of a pre-designated square (shown as a black square, zone 1–5) [i.e.,zone 1–5]. The Euclidean distance angle (EDA) for zone 1–5 was defined as  $\phi_i$ ,  $i = 1–5$ . (B): Defining diffusion flux angle (DFA). (a) Diffusion flux in a Xona™ XC450. Green squared area show a somal and neurotrophin compartment, which are magnified in (b). (b) Magnified image of diffusion flux of Zone 1–5 in a Xona™ XC450. Orange highlighted zone (zone 4) was magnified at the bottom of (b), defining a DFA ( $\psi$ ).

427 esized here that cell orientation is directionally controlled by the flux vector  
428 which is driven by the concentration gradient. Figure 8B shows the flow vectors  
429 in a somal compartment at Day 7 computed by COMSOL Chemical engineering  
430 module. We averaged the 10 flow vectors in each of five zones in Figure 8 to  
431 compute diffusion flux angle (DFA),  $\psi_i$ , where  $i = 1\text{--}5$  in Figure 8. To lighten  
432 the computational intensity, we reduced a dimension from 3D to 2D to com-  
433 pute diffusion Flux. Please see justification in Supplementary Data. All of the  
434 computed EDAs and DFAs can be found in Supplementary Table 2.

435 Figure 9 shows time-series of microscopic phase-contrast photomicrographs  
436 obtained on Day 1, 3, and 7 in the five zones in a Xona™ XC450. Each preferred  
437 orientation of any given cell was computed and then plotted on a polar diagram  
438 (blue circle). Mean vector angle (MVA, shown in red line on Figure 9) and  
439 median vector angle were computed. All of the polar diagrams in Figure 9 show  
440 that preferred orientation of hPSC-derived ONPs distribute in an unimodal dis-  
441 tribution. We also confirmed that a von Mises distribution is appropriate for  
442 these sets of data (See Supplementary Figure S9). We, therefore, then tested  
443 further to see if the cells had tendency to be oriented to a certain direction.  
444 To test this hypothesis, we used the Rayleigh test of uniformity to evaluate  
445 whether there is statistical evidence of circular directionality [32]. Computed  
446  $p$  values for all the 15 conditions were less than 0.05, demonstrating that all  
447 of the conditions had statistically significant directionality. To further validate  
448 whether the observed angles have a tendency to cluster around the two hypo-  
449 thetical angles (i.e., EDA and DFA), we then performed the V test. Once again,  
450  $p$  values for all 15 conditions were less than 0.05 except one (Zone 5 on Day 1),  
451 re-demonstrating that most of the conditions had statistically significant ten-  
452 dencies to cluster around the EDAs and DFAs. Finally, to investigate whether  
453 the preferred orientation of the cells were clustered around the EDAs or DFAs,  
454 we performed one sample test for the mean vector angle, which is similar to a  
455 one sample t-test on a linear scale. There was only one condition (Zone 1, day  
456 1) that was statistically significant for EDA, whereas most of the conditions on  
457 Day 3 and 7 were statistically significant for DFA. Therefore, our results here

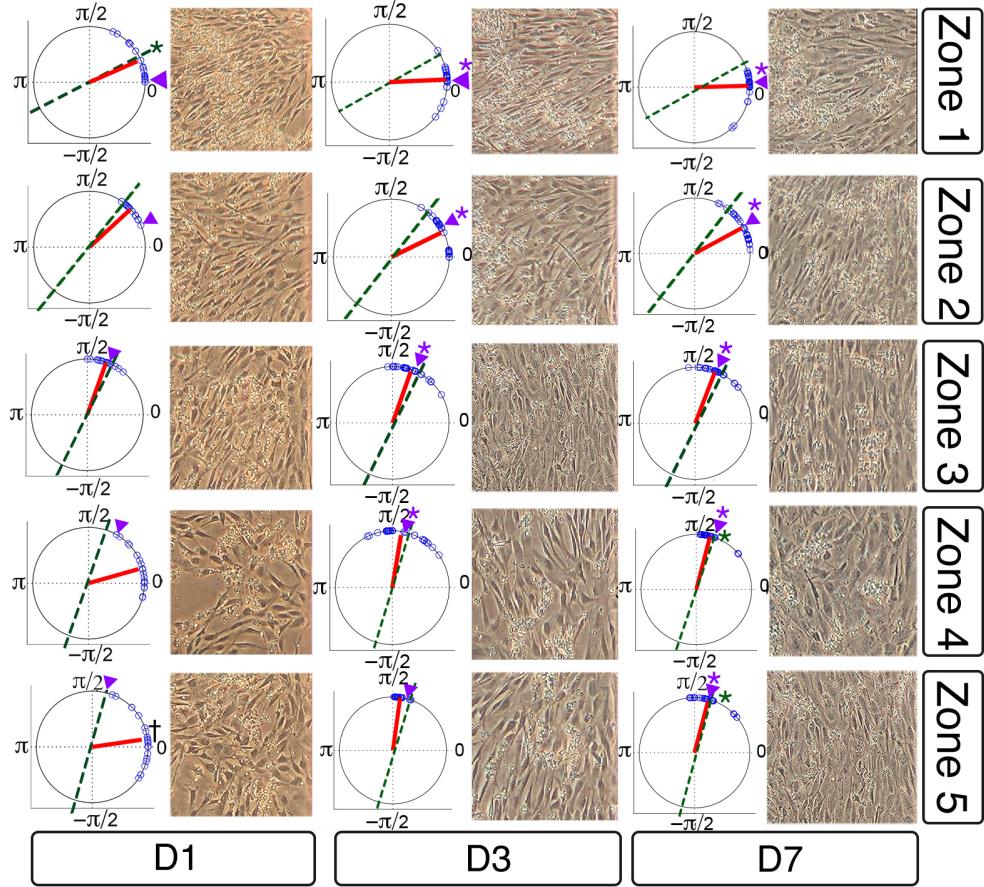


Figure 9: Preferred cell orientation analysis. Sequential phase-contrast images of a somal compartment of a Xona™ XC450 device in zone 1–5 (see Figure 8) on day 1 (D1), day 3 (D3), and day 7 (D7). A corresponding polar histogram indicates preferred orientation of hPSC-derived ONPs in an angle ( $0$ – $2\pi$  radians) in blue circles. Mean vector angle is shown in red line. Green dotted line shows Euclidean Distance Angle (EDA) and purple triangle indicates Diffusion Flow Angle (DFA). † symbol indicates that  $p$  value  $> 0.01$ . \* symbol indicates that the mean vector angle fits within the 95% confidence internal ( $\alpha < 0.05$ .)

458 demonstrated that hPSC-derived ONPs had greater tendency to cluster around  
459 DFA than EDA. All computed statistical values are shown in Supplementary  
460 Table S2.

461 To evaluate the direction of neurites of hPSC-derived ONPs, we first de-  
462 fined EDA in Region 1-3 ( $\phi'_j$ ,  $j = 1 - 3$ ) (Figure 10A) and DFA ( $\psi'_j$ ,  $j = 1 - 3$ );  
463 similarly defined  $\phi_i$  and  $\psi_i$  as in Figure 8. All of the EDAs and DFAs defined  
464 here can be found in Supplementary Table S3. Polar histograms of the neurite  
465 direction angle in Region 1–3 indicated that the two longest neurites were bi-  
466 modal in nature (Figure 10B). In contrast, polar histograms of those cultured  
467 with rhBDNF (negative control) and 800,000 PODS®-BNDF (positive control)  
468 did not indicate bimodal distribution—the neurites did not show directionality  
469 (Figure 10C). The Rayleigh test of uniformity for both the positive and negative  
470 control were greater than 0.05, demonstrating that both of the conditions had  
471 no statistically significant directionality (Supplementary Table S3: highlighted  
472 in green). We also analyzed the direction of the neurites using circular statistics.  
473 To obtain more realistic mean vector angles, we doubled each angle and reduced  
474 the multiples modulo 360°. In circular statistics, the bimodal distributed data  
475 can be transformed into a unimodal data by doubling the angle [32]. The mean  
476 vector angles in Figure 10D (right column) indicates the situation where the  
477 vectors were canceled out between the two groups of angles distributed in a bi-  
478 modal fashion, resulting in inaccurate representation. A circular plot in Figure  
479 10D (right column) showed doubled angles, representing actual representation  
480 of the neurite vector angles. In all of the three regions, the Reyleigh test and V  
481 test for EDA and DFA indicated directionality (Supplementary Table S3). One  
482 sample test for the mean vector angles in Region 1–3 indicated that they were  
483 not statistically different from DFA, but all of the three mean vector angles were  
484 statistically different from EDA.

485 We also stained the devices for  $\beta$ -III tubulin to track neurite growth and  
486 extension across the micro-groove channels as well as cell migration in three  
487 selected regions (Figure 10A). The location of the PODS®-rhBDNF disk in  
488 relation to the regions of interest in Figure 11A is indicated by a yellow circle.

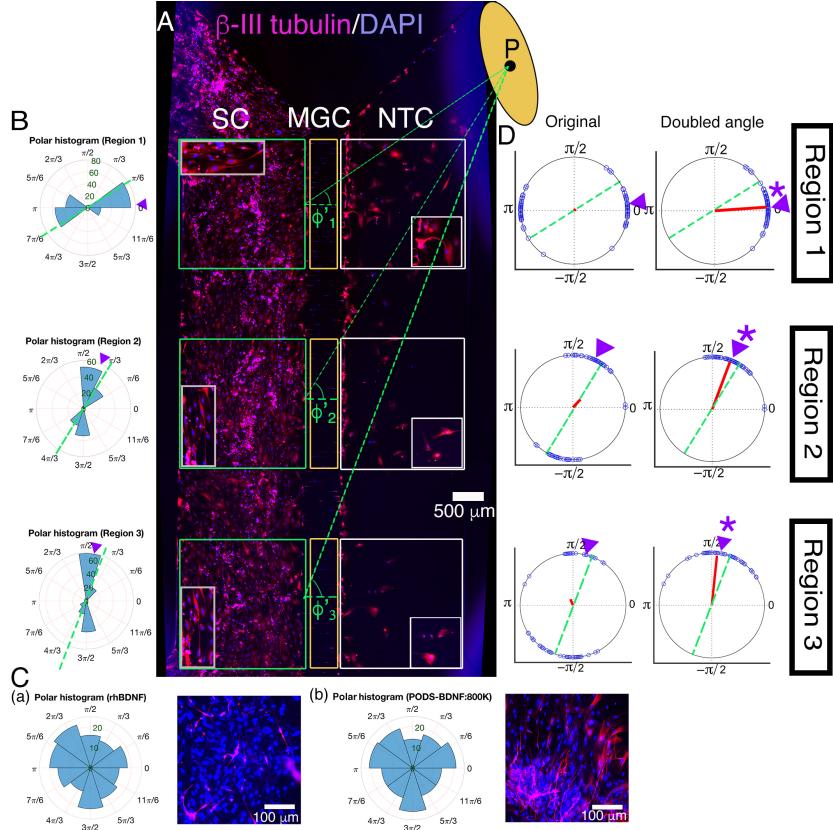


Figure 10: (A): Immunohistochemistry of hPSC-derived ONPs cultured in the Xona™ XC450 for seven days with  $\beta$ -III-Tubulin (pink fluorescence) and DAPI (blue fluorescence). A green line was drawn from the center of the BDNF disk (P) to the mid point of each of three pre-determined squares (Region 1–3) in the somal compartment to define Euclidean Distance Angle (EDA:  $\phi_i^c$ ,  $i = 1 - 3$ ). Green square: somal compartment (SC); yellow square: microgroove channels (MGC); White square: Neurotrophin compartment (NTC). High-power magnified images of the NTC and SC are shown. An ellipse-shaped PODS®-rhBDNF disc is shown in yellow. P: the center of the disk.(B): Polar histogram of neurite directional angles of hPSC-derived ONPs in the Xona™ XC450 at Day 7 (D7). The two longest neurites were measured. EDA: green dotted line. DFA: purple triangle. (C): (a) Right: Polar histogram of neurite directional angles of hPSC-derived ONPs cultured with 20 ng/mL of recombinant human BDNF for seven days. Left: Corresponding photomicrograph of immunocytochemistry with  $\beta$ -III tubulin (pink) and DAPI (blue). (b) Right: Polar histogram of neurite directional angles of hPSC-derived ONPs cultured with 800,000 of PODS®-rhBDNF for seven days. Left: Corresponding photomicrograph of immunocytochemistry with  $\beta$ -III tubulin (pink) and DAPI (blue). (D): A circular plot of neurite directional angle of hPSC-derived ONPs in Region 1–3. Once again, the two longest neurites were plotted on a unit circle. Right column: Original directional angle distribution. Left column: the method of doubling the angle applied to the original data. Each datum point is shown as a blue circle. Mean vector angle is shown as a red line. Green dotted line shows Euclidean Distance Angle (EDA) and purple triangle indicates Diffusion Flow Angle (DFA). \* symbol indicates that the mean vector angle fits within the 95% confidence internal ( $\alpha < 0.05$ .)

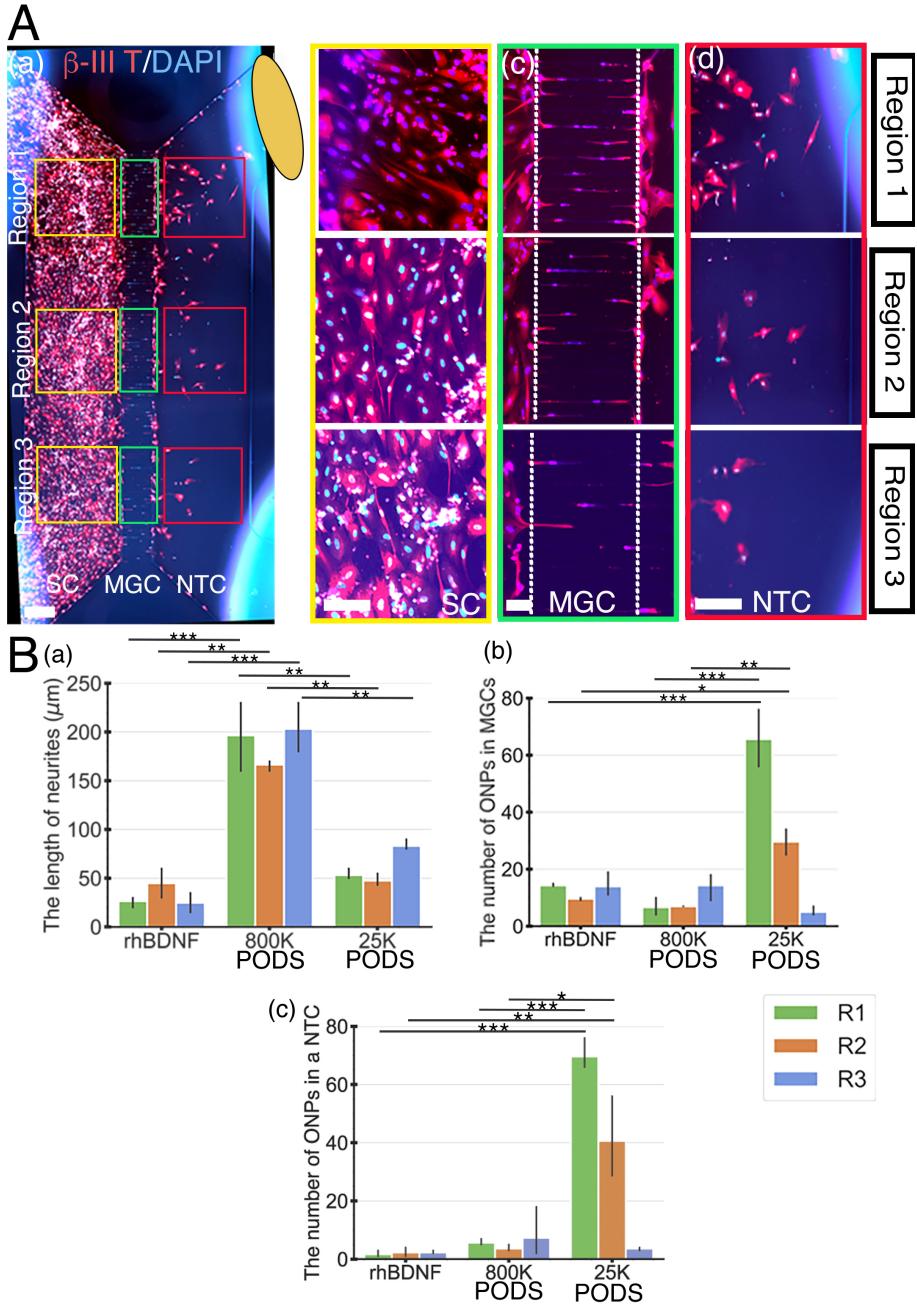


Figure 11: Human PSC-derived late-stage ONPs in the microfluidic device were stained with  $\beta$ -III tubulin (red) and DAPI (blue) to measure cell migration. (A): Late-stage ONPs stained after seven days in culture (a) with regions highlighted and magnified in the somal compartment (b), microgroove channels (c), and neurotrophin compartment (d). SC: somal compartment, MGC: microgroove channels, NTC: neurotrophic compartment. An ellipse-shaped PODS®-rhBDNF disc is shown in yellow. Scale bar (a): 500  $\mu\text{m}$ ; (b)–(d): 100  $\mu\text{m}$ . (B): Quantitative analyses of cell migration and neurite extension in devices using recombinant human BDNF, 800,000 PODS®-rhBDNF, or 20,000 PODS®-rhBDNF. \* :  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ .

489 Quantitative analyses were performed and summarized in Figure 11B. Our data  
490 indicate that neurite length is dependent on BDNF concentration, with greater  
491 amounts of PODS<sup>®</sup>-rhBDNF promoting longer neurite growth (Figure 11B(a)).  
492 Lesser amounts of PODS<sup>®</sup>-rhBDNF, however, are necessary to create an ap-  
493 propriate concentration gradient. In the presence of 20,000 PODS<sup>®</sup>-rhBDNF,  
494 both neurite extension into the microchannels and cell migration into the neu-  
495 rotrophin compartment are greatest in the region closest to the BDNF source  
496 and decrease further from the PODS<sup>®</sup>-rhBDNF (Figure 11B(b,c)). Cell migra-  
497 tion is dependent on the distance from the source of BDNF, thus suggesting the  
498 presence of a BDNF gradient as predicted by our model. Note that the Xona  
499 microchannels intended to prevent from migration across channels.

500 **4. Discussion**

501 *4.1. Challenges in neurotrophin treatment in the inner ear*

502 This is a proof-of-concept study for the realization of a neurotrophic strip  
503 to ascertain its scientific/technological parameters in a controlled *in vitro* en-  
504 vironment. Neurotrophin gradients have been studied for in multiple contexts  
505 [47, 48, 49]. However, it has not been feasible to reliably provide such a gra-  
506 dient to neurons neither *in vitro* nor *in vivo*, primarily because of technical  
507 challenges. While neurotrophin treatment has been recognized as a potential  
508 treatment for sensorineural hearing loss, there has not been clinical success in  
509 this avenue to date. Most recent relevant clinical trials used adeno-associated  
510 virus (AAV2) to deliver BDNF to the brain [50]. Although exciting, this treat-  
511 ment does not attempt to control the concentration of BDNF, which could  
512 potentially interfere with normal functions in a target organ [51]. Furthermore,  
513 this treatment may not be applicable to the inner ear. In this study, we used  
514 PODS<sup>®</sup>-rhBDNF to provide a neurotrophic gradient in a controlled fashion.  
515 Our result indicated 20,000 PODS-BDNF allowed for rhBDNF neurotrophin  
516 gradient such that hESC-derived ONPs survived, differentiated toward human  
517 SGNs, and also established directional neurite outgrowth in a microfluidic device.

518     4.2. Microfluidic device-generated gradient

519     We used a microfluidic device to advance our understanding of directional  
520     neurite growth and otic neuronal differentiation in response to a BDNF concen-  
521     tration gradient [52]. Among many *in vitro* concentration gradient sustaining  
522     culture devices, microfluidic devices have overcome many of the deficits that  
523     conventional platforms (i.e., the Boyden chamber, Dunn chambers, or compart-  
524     mentalized diffusion chambers) face [52]. Conventional platforms tend to be sub-  
525     optimal to manipulate small volumes of fluid at the order of microliters. Growth  
526     factors and proteins are used in minute amounts in our microfluidic device, and  
527     cultured stem cells are able to interact with endogenous factors. As mentioned  
528     earlier, this micro-environment more accurately represents *in vivo* conditions.  
529     The Xona™ device can be used to sustain a three-dimensional concentration gra-  
530     dient over time (duration dependent on the half-life of the molecule) due to its  
531     microchannel array. The device limits convective flow in the gradient-forming  
532     areas by introducing microgroove channels that generate high fluidic resistance,  
533     thereby limiting flow to diffusion. The high resistance of the microchannel array  
534     also prolongs diffusion across them, thereby increasing both gradient formation  
535     and gradient steepness. These features allowed us to generate a FEM, which pre-  
536     dicted the necessary number of PODS®-BDNF crystals for a BDNF gradient.  
537     Note, however, that this environment is different from the micro environment  
538     in the inner ear. A mesh geometry of the cochlea will be needed to compute the  
539     PODS®-BDNF crystal number for our next step of this study.

540     4.3. BDNF and Polyhedrin protein

541     Over the course of past 20–30 years, it has been established that BDNF me-  
542     diates survival and differentiation activities on SGNs by binding and activating  
543     the tyrosine kinase receptor kinase B (TrkB), a member of the larger family  
544     of Trk receptors [20]. Numerous studies have reported that BDNF can palliate  
545     SGN degeneration in ototoxically deafened animals, a widely accepted model for  
546     retrograde trans-synaptic SGN degeneration secondary to hair cell destruction

[13, 14, 53, 54]. Additionally, it has been confirmed that there is a positive correlation between SGN counts and CI performance [55]. It is then safe to presume that treating CI recipients with BDNF would enhance overall CI performance, by preserving SGNs and their neurites. However, simply introducing rhBDNF into the inner ear poses significant hurdles. Although promising, human BDNF treatment has not been currently implemented in the inner ear. Unsuccessful BDNF treatment can be explained by several factors [56].

The blood half-life of BDNF protein is extremely short, only 1–10 min in the plasma [57, 58] and one hour in CSF [59]. The BDNF's high degradation rate would require continuous replenishment, impractical for clinical use. Furthermore, introduction of a homogeneous solution of BDNF would promote nondirectional neurite growth where directed neurite growth is essential for designing our new-generation bioactive CI, as depicted in Figure 1A. Directing neurite growth towards the CI electrode array is pivotal in the ultimate goal of enhancing performance through the narrowing of the electrode-neuron gap. The PODS<sup>®</sup> system precludes the phenomena by its localized, gradual release of growth factor. The steady supply of BDNF from a localized origin not only creates a concentration gradient, but maintains it over time. As seen in Figures 4–6, we were able to perform a finite element analysis based on data we collected describing the chemical release kinetics and molar ratio of PODS<sup>®</sup>-BDNF system. It is clearly visible that the slow-release nature of PODS<sup>®</sup>-BDNF results in a concentration gradient over the course of Day 1–7 (Figure 5).

It should be noted that our FEM assumes free diffusion of the rhBDNF protein. In biological cell-culture conditions, BDNF released from PODS<sup>®</sup>-BDNF has tendency to adhere to the walls of the culture device because BDNF is a “sticky” protein of about 27 kDa (mature BDNF dimer) and it is positively charged under physiological conditions (isoelectric point, pI = 9.4) [60]. As such, the physio-chemical properties of BDNF have made the recombinant protein difficult to diffuse. This phenomenon was observable in preliminary data where the ONPs failed to survive past 1–3 days of culture (data not shown). To circumvent this issue we infused the culture media with a carrier protein (i.e.,

578 BSA), hypothesizing that the albumin would act as a carrier for the released  
579 BDNF and allow for free diffusion throughout the microfluidic device [61]. This  
580 hypothesis is supported by our sets of biological verification data (Figures 7–11)  
581 that clearly shows that hPSC-derived ONPs responded to the modification by  
582 exhibiting the expected cell body orientation, unidirectional neurite extension,  
583 and neurite length. Note that albumin is the single protein found in highest  
584 concentrations in the perilymph [62], therefore, a carrier protein will not be  
585 needed in our future *in vivo* study.

586 *4.4. Intracellular signaling initiated by Thyrosine kinase B receptor*

587 Another issue we need to consider in interpretation of our results is the intra-  
588 cellular cell signaling mechanism elicited by rhBDNF. Human BDNF (mature  
589 dimeric form) binds with high affinity to its TrkB receptor. The binding of  
590 BDNF to a TrkB receptor has proven to have significant importance for the  
591 proneuronal effects of BDNF [20]. Upon binding BDNF, TrkB dimerizes, and  
592 activates intrinsic kinase activities and other complex set of intracellular sig-  
593 naling cascades, which is beyond the scope of this study. However, it should  
594 be noted that activation of TrkB receptor by neurotrophin binding causes the  
595 TrkB protein to be internalized in endosomes on the cellular membrane [63].  
596 Endosomes can then be transported to the soma. Therefore, the proneuronal  
597 effects of rhBDNF in our results might have highly depended on the status of  
598 TrkB receptors on the cell membrane of hPSC-derived ONPs. Our previous  
599 study has demonstrated that strong expression of a TrkB receptor on derived  
600 ONPs [24], however, more detailed studies on TrkB receptors on hPSC-derived  
601 ONP and SGNs will be needed.

602 *4.5. Degradation of PODS® crystals by protease*

603 In cell culture, degradation of PODS®-rhBDNF is likely due to the activity  
604 of cell-secreted proteases. The proteases break down the peptide bonds of the  
605 encasing polyhedrin protein, creating openings in the structure to allow release  
606 of the rhBDNF. Therefore, the presence of proteases is imperative for the proper

607 utilization of the PODS<sup>®</sup> crystals. Additionally, these proteases are responsible  
608 for the degradation of the released BDNF. Because stem cells are not present  
609 in the culture media used for the PODS<sup>®</sup> degradation kinetics experiments, we  
610 infused the media with 10% FBS, which naturally contains proteases, to promote  
611 polyhedrin degradation, BDNF release, and BDNF degradation to attain results  
612 that more accurately describe *in vitro* events. Moreover, since the cells and  
613 PODS are initially segregated into separate compartments within the culture  
614 device, cell-secreted proteases are unlikely to reach and degrade the PODS in  
615 time to support ONP survival and differentiation. Infusion of FBS was therefore  
616 required in these experiments as well. In clinical use, however, we presume  
617 that cell-secreted proteases will be readily present in the inner ear and would  
618 therefore preclude the need for artificial supplementation.

619 *4.6. A concept design: Neurotrophic strip*

620 The plateau in CI performance in treatment of sensorineural hearing loss has  
621 driven researchers to develop innovative supplementary treatment strategies to  
622 push the field past this hurdle. Our approach strives to directly address the  
623 issue at its core: the electrode-neuron gap, which can lead to serious implica-  
624 tions include low spatial frequency resolution and high power consumption. We  
625 can use our data as a launchpad for the neurotrophic strip (NS). The NS is a  
626 biointerface concept that integrates an extended-release source of growth factor  
627 to facilitate a protein gradient. Implanted in conjunction with the CI, it acts  
628 as a bridge between the extant SGNs and implanted late-ONPs grown on the  
629 electrode itself. The NS would promote survival of both cell populations, dif-  
630 ferentiation of the late ONP implants, promote directional neurite growth and  
631 synaptogenesis between the two, effectively creating a neuronal network between  
632 the patient and the implanted CI. Each electrode would be able to stimulate cell  
633 bodies at exceptionally high resolution, essential for greater intonation differ-  
634 entiability (required for effective social interaction and music appreciation) and  
635 so, increased quality of life for millions. Our successful outcomes are essential  
636 to make a neurotrophic strip feasible in *in vivo* environment.

637     *4.7. The limitations of this study and future direction*

638     There are some limitations associated with this study. First, the reduction of  
639     spacial dimension to 2D for diffusion modeling certainly affected the flux vector,  
640     which determines the predicted concentration vector. Given that the thickness  
641     (i.e., Z-axis) of the microfluidic device was 100  $\mu\text{m}$ , we estimated that the effect  
642     was minimal. In the future, we plan to use PSC-derived 3D spheroids in a somal  
643     compartment so that flux vector and concentration gradient vector can more  
644     accurately model the cell behavior. In this way, we will be able to circumvent  
645     the need to reduce diffusion calculations to 2D for computation performance in  
646     the modeling.

647     Secondly, we required to generate a model in that the BDNF's biological  
648     transportation phenomenon from a PODS<sup>®</sup>-rhBDNF disk to a somal compart-  
649     ment of a Xona<sup>TM</sup> device. Note that in this model, we focused on the major  
650     dependent variable, BDNF concentration gradient to model the biological phe-  
651     nomenon. Other physical variables to promote cell migration, otic neuronal  
652     differentiation, and neurite growth were not take into consideration. These  
653     variables include electotaxis (electrical potential), durotaxis (matrix's stiffness  
654     [i.e., laminin 511 in our case]) [64], mechanotaxis (cell strain), and lastly cell  
655     migration by random walk [65]. In our future study, we will take these vari-  
656     ables into consideration to more accurately represent the migration and neurite  
657     growth of hPSC-derived ONPs.

658     Insufficient contrast between cells and background in phase contrast images  
659     led to inaccuracies in cell orientation computation for some images. To address  
660     this issue, poor quality images were disregarded in the quantitative analysis.  
661     We occasionally used manual measurement for accuracy. Our future study may  
662     entail automated time-series cell analysis, which would allow more accurate  
663     measurement. Also, another way to address this issue would be with a cell  
664     membrane staining in the future.

665     While 20,000 of PODS<sup>®</sup>-rhBDNF were necessary for hPSC-derived ONPs  
666     for otic neuronal differentiation and directional neurite outgrowth, this condi-  
667     tion may not be sufficient. For instance, it is still not known whether the effects

668 of other neurotrophic factors such as Neurotrophin-3 and Glial cell line-derived  
669 neurotrophic factor could have a positive effect on ONP growth [20, 66, 67]. We  
670 are planning to investigate these neurotrophic factors in the future. Other fac-  
671 tors that could have an impact on directional neurite growth include endogenous  
672 factors secreted from hPSC-derived ONPs. While our previous study demon-  
673 strated that hPSC-derived ONPs only secreted negligible amount of BDNF that  
674 were detected by ELISA [9], currently we do not have any data on other neu-  
675 rotrophic factors or other molecules that could have affected directional neurite  
676 growth in the inner ear. We chose BDNF first to study because the most inten-  
677 sively studied neurotrophic factor in the field of hearing research is BDNF [20].  
678 Previous studies have indicated that neurotrophic supports of SGNs are mainly  
679 from BDNF and neurotrophin-3 (NT-3) [20, 60]. Therefore, the potential con-  
680 founding effect of the secretions of other neurotrophic factors and molecules  
681 secreted from derived SGNs are likely NT-3, for which further investigation is  
682 necessary in the future.

683 Despite the aforementioned limitations associated in this study, the present  
684 results generated BDNF concentration gradient, condition of which is necessary  
685 for hPSC-derived ONPs to differentiate into the otic lineage (i.e., SGNs) and  
686 also promoted directional neurite extension towards the POD-BDNF disk. The  
687 technique will allow us to control neurite direction of transplanted hPSC-derived  
688 ONPs in the inner ear. We will harness this method in our design of a bioactive  
689 CI.

## 690 **Conclusions**

691 We were able to generate BDNF concentration gradient, enabling survival,  
692 neuronal differentiation toward SGNs, and directed neurite extension of hPSC-  
693 derived ONPs. The technique will allow us to control neurite direction of trans-  
694 planted hPSC-derived ONPs in the inner ear. This proof-of-concept study pro-  
695 vides a step toward next-generation bioactive CI technology.

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