Supplementary Information:

3D Printed Nervous System on a Chip

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This supplementary information contains seven additional figures (**Figures S1-S7**) describing: 1) the 3D printing of cells, 2) rapid prototyping of the 3DNSCs, 3) scanning electron micrograph characterization of 3D printed microchannels on protein-coated substrates, 4) comparison of viral transport imaging on different substrate materials, and 5) association of Schwann cells and axons within microchannels.

1) Cell printing: Cell suspensions of hippocampal and SCG neurons were prepared following dissociation procedures. Cell suspensions of Schwann and epithelial cells were prepared by resuspending actively growing cells in fresh growth medium. The suspension was then aspirated to further break up cell clusters. Following preparation, the cell suspensions were loaded into extrusion syringes. Prior to printing, the tips were incubated in a blocking solution consisting of 10% bovine serum albumin (BSA, Sigma) in DPBS to prevent cell adhesion to the inner wall

of the tip. Cell suspensions were then printed into the individual chamber wells. As shown in **Figures S1**a-b, a complete 3DNSC involves an assembled substrate (**Figure S1**a; corresponding computer model) and tri-chamber (**Figure S1**b; corresponding computer model). As shown in **Figure S1**c, cell suspensions were printed using a 27 GA tapered plastic tip. Following printing, cell viability was analysed using a vital dye (Erythrosin B stain, ATCC). The viability was calculated as the number of live cells / the total number of cells × 100. Cells dispensed with a standard 1 ml pipette served as the positive control. Cell suspensions containing sodium azide served as the negative control.

As shown in **Figure S1**d, we found that the micro-extrusion process caused only small decreases in cell viability among both the primary cells and cell lines. 3D printed primary hippocampal and SCG neurons were 96% and 88% viable, respectively, while 3D printed Schwann cells and PK-15 cells were 97% and 96% viable, respectively. We attribute the mechanism of cell death to extrusion-associated shear stress, as a Newtonian fluid moving through a tapered pipe experiences losses according to the following relation:¹

$$\Delta p = \left(\frac{8\mu Q}{\pi}\right) \int_0^L \frac{dL}{R(L)^4} \tag{1}$$

where Δp is the pressure drop, L is the length of the extrusion tip, μ is the viscosity, Q is the flow rate, and R(L) is the length dependent radius. Viability studies performed using dead cells served as negative controls. Importantly, this result suggests that 3D printing could play a role as a valuable tool for the automated assembly of neurons, glia, and terminal cells.



Figure S1. a) Computer model of the inter-chamber 3D printed microchannels for axonal guidance (typical channel width = $350 \ \mu m$). b) Computer model of the 3D printed tri-chamber for cell isolation and organization. c) Photograph showing the printing of a cell suspension (extruder tip length (Lt) and tip diameter (Dt)). d) Viability studies on printed cells including hippocampal neurons, superior cervical ganglia (SCG) neurons, Schwann cells, and PK-15 cells (epithelial cells).

2) Rapid prototyping: In Figures S2-S5, we highlight the flexibility provided by 3D printing to customize and prototype the chip design in terms of altering: 1) the microchannel size (200–400 μ m wide parallel channels, as shown in Figures S2a-c), 2) the microchannel substrate material (glass), as shown in Figure S3a, 3) the 3D printed microchannel material

(polycaprolactone; data not shown), 4) the microchannel geometry, as shown in **Figure S3**b, which enabled the convergence of axons onto a pre-integrated TEM substrate (see Section S3-Electron Microscopy for information on preparation and handling), and 5) the tri-chamber geometry (symmetric and asymmetric) as shown in **Figures S3**c and d, respectively. We also include videos of the microchannel and tri-chamber printing processes as shown in Supplementary **Movies S1** and **S2**, respectively. Such flexibility provided by 3D printing enabled the nanoscale discrimination of single axons, which has future importance for the imaging of synapses and other close cell-cell contacts in the nervous system.



Figure S2. Representative scanning electron micrograph of the 3D printed microchannels, showing a filament of 3D printed material on a protein-coated substrate, which creates a network of parallel coated channels for cell confinement and axonal guidance with layer spacing of (a) 200, (b) 300, and (c) 400 μ m.



Figure S3. a) Demonstration of microchannel printing on alternative substrate materials (glass; inset shows assembled tri-chamber on a glass-based 3DNSC; inset scale bar = 10 mm). b) Photograph of substrate-integrated TEM grid chips on glass substrates, with converging microchannels and direct printing onto pre-integrated TEM scaffolds. c) 3D printed symmetric top tri-chamber (silicone) for integration with either plastic or glass substrates. d) 3D printed asymmetric top tri-chamber (silicone).

3) Electron microscopy: Samples were prepared for electron microscopy as previously described.^{2,3} Immediately after fluorescence microscopy, axons were fixed in 4% PFA and 2.5% glutaraldehyde in Sorenson's buffer (0.1 M sodium phosphate buffer, pH 7.4) for 6 hr. Samples were then rinsed in clean buffer, post-fixed with 1% OsO4 in Sorenson's buffer for 30 min, and rinsed again. Axons were permeabilised by rinsing in 15% ethanol (v/v in water) followed by staining with 2% uranyl acetate in 15% ethanol for 5 min. Samples were then rinsed with 15% ethanol, and dehydrated in a series of ethanol washes: 30%, 50%, 70%, 90% (5 min each), followed by five washes in 100% ethanol. Samples were rinsed twice for 10 min each in hexamethyldisilazane, and allowed to air dry overnight. Samples were imaged on a Philips CM100 electron microscope at the Imaging and Analysis Centre of the Princeton Institute for the Science and Technology of Materials.

The 3D printing approach enabled us to readily modify the platform design in order to more closely interrogate particular components of the nervous system (e.g. single axons) as shown in **Figures S4** and **S5**. Cellular communication in the nervous system occurs via axons, so it is critical to demonstrate that the 3DNSC design could be modified to accommodate advanced imaging of axons. In general, it is challenging to control and optimize the growth of axons on materials for advanced imaging applications, such as grids for electron microscopy applications. Thus, the microchannel design was modified from a parallel to a converging configuration in order to guide the axons on a substrate-integrated transmission electron microscopy (TEM) grid (see **Figure S3b**). As shown in **Figure S4**a, the TEM-modified peripheral 3DNSCs were constructed by functionalizing chamber 1 with SCG neurons and integrating a TEM grid in chamber 3. Chamber 2 was left empty to enable high resolution imaging of the converging microchannel configuration

effectively concentrated axons in the TEM grid region (**Figure S4**b). The axons also spontaneously grew on top of the TEM grid itself, as shown in **Figure S4**c. We observed a concentration of axons of ca. 15 axons per TEM grid element ($100 \times 100 \ \mu m^2$). As shown in **Figure S4**d, this 3D printing approach enabled the high resolution imaging of single axons using electron microscopy, via this ability to interface biological systems (e.g. axons) and materials (e.g. TEM grids).



Figure S4. a) Micrograph of a customized 3DNSC with a converging microchannel design, which concentrates axonal growth towards a substrate-integrated TEM grid. b) Enlarged view corresponding to (a) showing guidance of axons to the substrate-integrated TEM grid. c) Enlarged view corresponding to (b) showing axons which have grown spontaneously on top of the TEM grid. d) Electron micrograph of a single axon which has been guided onto the TEM grid by the converging microchannel design.

4) Viral imaging: In addition to the enhanced imaging of nervous system components (e.g. axon pathways) using electron microscopy as shown in Figure S4, the ability to rapidly prototype 3DNSC designs according to the strategies shown in Figure S3 also enables enhanced fluorescence imaging applications. Specifically, we compare the signal quality in terms of signal-to-noise ratio of total internal reflectance fluorescence microscopy measurements taken from 3DNSC's fabricated both on glass (Figures S5a and b) and plastic (Figures S5c and d). The results shown in Figure S5 show that significant improvement in signal-to-noise ratio was achieved on glass substrates which enabled the high resolution real-time tracking of individual virus particles (indicated by white arrows in Figure S5).



Figure S5. Comparison of fluorescence images of viral transport in infected SCG neurons on glass (a and b) and plastic (c and d) substrates at t = 0 and 2 s, highlighting the ability to optimize the measurement conditions with the flexibility in material assembly provided by the 3D printing process.

5) Schwann Cell-Axon Association Analysis: The associated phase contrast micrographs of Figure 2 are presented in Figure S6. Figures S6c and S6f were maintained to preserve the multichannel viral fluorescence, primarily of a single type, indicating infection by single viral particles.



Figure S6. a) Schematic of a peripheral 3DNSC showing three primary components: (1) the neuronal component and source of axons containing SCG neurons in chamber 1, (2) the peripheral nerve component containing axons and Schwann cells in chamber 2, and (3) the terminal cell junction component containing axon termini and epithelial cells in chamber 3. b) Micrograph showing three parallel microchannels of SCG neurons and axons in chamber 1. c) Micrograph showing three parallel channels of peripheral nerve fibres containing organized self-assembled networks of axon-associated Schwann cells (tri-colour PRV Brainbow strain; see Materials and methods section) in chamber 2. d) Micrograph showing three parallel channel image of neurons from (b). f) Single channel image of Schwann cells from (c). g) Single channel image of axon termini and epithelial cells from (d).

We also analysed the association between the Schwann cells and bundling axons in the microchannels. As shown in Figure S7, repeated studies at relatively lower Schwann cell densities enabled the characterization of Schwann cell distributions in channels both with and without axons present (Figures S7a and S7b, respectively). Figure S7c shows that Schwann cells were randomly distributed across the entire channel when axons were absent, but were localized when axons were present to the region of the axon. This result suggests that Schwann cells associate with axons, although the mechanism is beyond the scope of this work.



Figure S7. Representative phase contrast micrographs of Schwann cell microchannel growth both without (a) and with (b) axons present. c) Histogram of the Schwann cell microchannel distribution, where y is the distance from the top channel wall and W is the channel width.

Movie S1. Movie of the microchannel printing process on glass substrates (× 16 speed).

Movie S2. Movie of the tri-chamber printing process (× 64 speed).

Supporting References

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