Supplementary Materials for

**Three-dimensional, multifunctional neural interfaces for cortical spheroids and engineered assembloids**

Yoonseok Park, Colin K. Franz, Hanjun Ryu, Haiwen Luan, Kristen Y. Cotton, Jong Uk Kim, Ted S. Chung, Shiwei Zhao, Abraham Vazquez-Guardado, Da Som Yang, Kan Li, Raudel Avila, Jack K. Phillips, Maria J. Quezada, Hokyung Jang, Sung Soo Kwak, Sang Min Won, Kyeongha Kwon, Hyoyoung Jeong, Amay J. Bandodkar, Mengdi Han, Hangbo Zhao, Gabrielle R. Osher, Heling Wang, KunHyuck Lee, Yihui Zhang, Yonggang Huang*, John D. Finan*, John A. Rogers*

*Corresponding author. Email: y-huang@northwestern.edu (Y.H.); jdfinan@uic.edu (J.D.F.); jrogers@northwestern.edu (J.A.R.)

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**Other Supplementary Material for this manuscript includes the following:**

(available at advances.sciencemag.org/cgi/content/full/7/12/eabf9153/DC1)

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Movie S2. Insertion of cortical spheroid into a 3D mesoscale multifunctional framework.

Movie S3. Representative firing and bursting events across the neural spheroid during 250 ms.
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Fig. S8 RNA-Seq measurements of gene expression levels. RNA-Seq measurements of gene expression levels in spheroidal cultures for genes specific to (A) cortical layer I, (B) cortical layer II/III and (C) cortical layer V/VI.
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Fig. S15. Electrically evoked responses across a cortical spheroid. (A) 3D map of 12 microelectrodes along the X-axis and Y-axis. (B) A 3D plot of the amplitude of field potentials along X-axis (top) and Y-axis (bottom) across the neural spheroid evoked at single electrode (number 19) with a potential of 5 mV (left), 10 mV (middle) and 50 mV (right). (C) Representative field potentials from 25 electrodes recorded during electrical stimulation.
Fig. S16. Schematic illustrations, optical images and modeling results for integration and operation of a µ-ILED in a 3D MMF. (A) Picture of a 3D MMF with an integrated blue µ-ILED in the OFF and ON state. (B) Schematic illustration of evaluation set-up using a digital power meter (PM100D; power meter and S120VC; photodiode sensor, Thorlab, NJ, USA) placed at different distances ($d = 0.7, 1, 1.5$ mm) relative to the µ-ILED. (C) Measured dependence of the optical power density on the applied electrical power and distance (in air). (D) Schematic illustration of steps for integration of an µ-ILED ($\lambda_p = 470$ nm, $270 \times 220$ μm, C470TR2227, Cree, NC, USA) into a 3D MMF. (E) Computational results for the optical intensity distribution around the µ-ILED (optical input power = 10 mW) and (F) between the µ-LED and the spheroid. Photo credit: Yoonseok Park, Northwestern University.
Fig. S17. **Optically stimulated responses from a cortical spheroid.** (A), Schematic illustration of optical stimulation using optical fiber ($\lambda_p = 470\text{nm};$ fiber-coupled LEDs, M470F3, Thorlab, NJ, USA) and (B) optical property evaluation ($8.35\text{ mW/mm}^2$) in air using a digital power meter (PM100D; power meter and S120VC; photodiode sensor, Thorlab, NJ, USA) placed at 500 µm. (C, D) Representative field potential trace recorded during optical stimulation ($P_{\text{input}} = 800\text{ mW, 1Hz}$). (E) Overlaid plots of evoked spikes from 25 channels.
Fig. S18. Design, fabrication and operation of an electrochemical oxygen sensor. (A) Schematic illustration of an electrochemical sensor that includes PtBk, bare Au and Ag/AgCl as working, counter and reference electrodes, respectively. (B) Cyclic voltammograms for different concentrations of oxygen 0, 5, 15%. (C) Schematic illustration of steps for integration of an electrochemical sensor into a 3D MMF.
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Fig. S20. Dimensional information for a set of complex 3D MMFs by FEA. Maximum height and lateral dimensions information of the 3D MMF (A) with a layout that accommodates three organoids, (B) with a full coverage across the entire 4π solid angle and (C) with deformable, serpentine structural elements.
Fig. S21. Key dimensional aspects of advanced 3D MMFs. Lateral dimensions information of the whole 3D MMF (A) with a layout that accommodates three organoids, (B) with a full coverage across the an entire $4\pi$ solid angle and (C) with deformable, serpentine structural elements.
Fig. S22. Optical micrographs of a 3D MMF with a high-density array of microelectrodes. Optical image of a high-density array of electrodes on a 3D MMF (9 electrodes in an area of 60 × 60 μm; electrode size 10 μm; distance between electrodes 30 μm). Photo credit: Yoonseok Park, Northwestern University.
Fig. S23. 3D MMF arrays. Optical micrograph and FEA modeling of 3D MMF arrays. Photo credit: Yoonseok Park, Northwestern University.
Supplementary Note 1.

Pre-plated, ready to use, 3D neurospheroids composed of cortical neurons and astrocytes were obtained from StemoniX, Inc. (microBrain 3D®, StemoniX Cat # BSARX-AA-0384). Neural spheroids were generated from human induced pluripotent stem cells (hiPSCs) as described previously(19,20). Briefly, neural progenitor cells (NPCs) were isolated from hiPSCs-derived neural rosettes(19), cultured in spheroid format, and matured into cortical neurons and astrocytes(20). RNA-Seq analysis demonstrates cortical identity through expression of cortical layer genes (RELN, NDNF, CNR1, POU3F3, and BCL11, see Fig. S7) while synchronized spontaneous activity along with astrocyte, neuronal, and synaptic markers demonstrate the presence of functional neural structure. These spheroids contain astrocytes and neurons at a ratio of about 1:1. The neurons express MAP2, indicating that they are mature, and synapsin I, indicating the presence of synapses. This data is consistent with the robust, spontaneous, synchronous, electrophysiological activity of these cultures, which demonstrates functional maturity and connectivity. Neurotransmitter receptor and ion channel genes are expressed in these spheroids at levels similar to their expression in human brain(21).

Upon receipt from the Manufacturer, the medium was changed (1/2 volume, 3 times) using BrainPhys Neuronal Medium SM1 Kit (StemCell Technologies, No. 05792, BC, Canada) supplemented with 20 ng/ml of recombinant human BDNF (StemCell Technologies, No. 78005, BC, Canada), 20 ng/ml of GDNF (StemCell Technologies, No. 78058, BC, Canada), and 1X Penicillin-Streptomycin (GE Healthcare Life Sciences, PA, USA) or the NeuralX Cortical Neuron media kit that follows a recipe published by Bardy et al.(44) (StemoniX, NXCNM-AA-0250, MN, USA). Spheroids were maintained at 37 °C and 5% CO2 until used in experiments. Half media changes were performed every Monday, Wednesday, Friday using one of the two above described media. Manufacturing batch consistency and media equivalency was confirmed as spheroid activity and size were highly reproducible across manufacturing lots and culture media (see Supplementary Table 1).
**Supplementary Note 2.**

Polyimide and parylene-C have comparable elastic modulus and Poisson’s ratio of $E_{\text{PI}} = 2.5$ GPa, $\nu_{\text{PI}} = 0.34$ and $E_{\text{parylene}} = 2.76$ GPa, $\nu_{\text{parylene}} = 0.4$, respectively, making the 3D devices made of either polyimide or parylene-C mechanically similar. Moreover, when the elastic substrate is sufficiently stiff (sufficient combination of thickness and elastic modulus), the 3D device is controlled and the final 3D geometry is independent of material elastic modulus of the 3D device; In our design, the elastomer substrate made of PDMS can be assumed sufficiently stiff compared with Polyimide and parylene-C.

**Supplementary Note 3.**

Tests of optical stimulation without the spheroid yield no measurable responses. Operation of the integrated $\mu$-ILED requires electrical isolation to avoid the introduction of noise in the metal traces for the electrodes. The optical fiber eliminates this source of noise.
**Supplementary Table 1** Spheroid reproducibility across manufacturing batches and culture media. Manufacturing batch represents independent production runs that were cultured in either NeuralX or BrainPhys culture media. Activity is measured as the number peaks shown by the synchronized, spontaneous, oscillatory spheroid activity measured as Ca2+ waveforms as previously described\(^{23}\) and expressed as the mean across at least XYZ test wells in each plate. Size was measured as spheroid diameter captured with XYZ microscope for all spheroids in the test 384 well plates from each manufacturing batch.

| Manufacturing Batch | Average Activity (peaks / 10 min.) | Average Spheroid Diameter (µm) |
|---------------------|------------------------------------|---------------------------------|
|                     | NeuralX | BrainPhys | NeuralX | BrainPhys |
| A                   | 8.3     | 4.1       | 649     | 681       |
| B                   | 5.6     | 4.2       | 653     | 697       |
| C                   | 5.2     | 9.3       | 625     | 691       |
| D                   | 6.8     | 4.3       | 695     | 586       |
| E                   | 4.6     | 6.6       | 662     | 676       |
| Mean +/- SEM        | 6.1 ± 1.3 | 5.7 ± 2.0 | 656.8 ± 11.3 | 666.2 ± 20.4 |
Supplementary table 2. Age and lot number of spheroids from Stemonix

| Spheroid | Age of Spheroid (Day 0, DaF 0) | lot number from StemoniX |
|----------|--------------------------------|--------------------------|
| 1        | 10w                            | 20190707-01              |
| 2        | 12w                            | 20190707-01              |
| 3        | 14w                            | 20190707-01              |
| Assembloid | 14w                        | 20190417-01              |
| 2        | 10w                            | 20190707-01              |
| 3        | 13w                            | 20190707-01              |