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Supporting Information

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Simple Precision Creation of Digitally Specified, Spatially Heterogeneous, Engineered Tissue Architectures

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Individual masks were designed for each colored section of the overall geometry which are referred to as elements of the digitally fabricated geometries. Masks were created using AutoCAD (AutoCAD, v2012, Autodesk Inc., CA), for 5 different geometries (square, concentric circles, radially fractioned circles, two consecutive squares with dimensions: 100 μm by 100 μm; 500 μm by 500 μm, and microscale script (BWH, BAMM, HST, MIT) (**Fig. s1**). Within each geometry, to create each of elements, different set of masks were designed. The number of masks for each geometry equaled the number of elements it has and each mask was labeled on top with a letter and a number that represents the geometry and the element, respectively. Every mask was designed such that when aligned on top of each other they would create consecutive or concentric shapes. Alignment of the masks with the mask holders was achieved through 4 reference points that are located at each corner of the mask and separated by 16 mm. The black frame in the mask was designed to be 15 mm by 15 mm.

To ensure precision in the alignment of the individual units of the digitally sculpted tissue prototypes, a dual system of holders for the mask and the coverslip were designed and fabricated. Masks were created for each element of a specific geometry were placed on separate holders and the four reference dots on them were aligned with the holes of the holder (**Fig. s2**) under a stereomicroscope (Leica Microsystems Inc., Buffalo Grove, IL). This alignment provided the basis for maintaining the desired precision. The distance between any two reference dots was 16mm. The labels on each mask were used to maintain consistency throughout the fabrication of the elements. Between the steps of fabricating each element, when the masks were swapped, each label was oriented towards a mark that was placed on the cover slip holder. A TMSPMA coated cover slip was placed in the holder and stabilized with a low- viscosity adhesive (Loctite 420 Instant Adhesive Wicking Grade, Henkel). Stabilizing the coated coverslip is significant in maintaining the precision in the alignment of each element. Once stabilized, a space of 150μm was created with a glass slide spacer (**Fig. s2**).

The alignment posts were used to align and keep the setup stable during the fabrications process. Once the masks were aligned on the mask holder the following procedure was followed for fabrication:

I. The first element of the tissue prototype was fabricated by pipetting 50 µL of the hydrogel precursor solution on to the 3-(trimethoxysilyl) propylmethacrylate (TMSPMA) (Sigma-Aldrich, St. Louis, MO, USA) coated glass slide which was then covered with a secondary coverslip (**Fig. 1 and Fig. s2**). In the case where a thicker shape was created as in the case of multilayer fabrication (300 µm height), 100 µL of hydrogel precursor solution was pipetted on to the coverslip.

II. The mask holder was placed on top of the coverslip holder and both were connected through the alignment posts. In this configuration, the mask was placed directly above the TMSPMA coated coverslip, allowing the cross-linked hydrogels to attach to the coated coverslip.

III. The setup was placed under the UV light using The Omnicure® S2000 UV/Visible Spot Curing System (EXPO Photonic Inc., Ontario, Canada). Mask holder side was oriented up, and the system was exposed to light from a distance of 35 mm. The power density was set to be 3.6 mW/cm² during the fabrication of colored tissue prototypes and at 2.5 mW/cm² for cell encapsulating tissue prototypes. The duration of the exposure varied according to the color of the hydrogel solution (**Table s1**).

IV. As the final step, the top coverslip was removed and the uncross-linked hydrogel precursor solution was washed away with PBS.

V. To fabricate the second element of tissue prototype, a second precursor solution was added to the same TMSPMA coated coverslip and was again covered with a second coverslip along with the first elements of the tissue prototype. The mask holder with the second mask (**Fig. s1**) was then placed on top of the coverslip holder and similarly,

was exposed to UV light. This process was repeated to get the desired tissue prototype. At the end of this process, a tissue prototype composed of digitally specified elements was fabricated (**Fig. 1**). The fabricated tissue prototype containing Procion MX dyes (Sigma-Aldrich, St. Louis, MO, USA) were imaged both with a camera (Sony, DSLR A700) and an inverted microscope (Nikon TE2000).

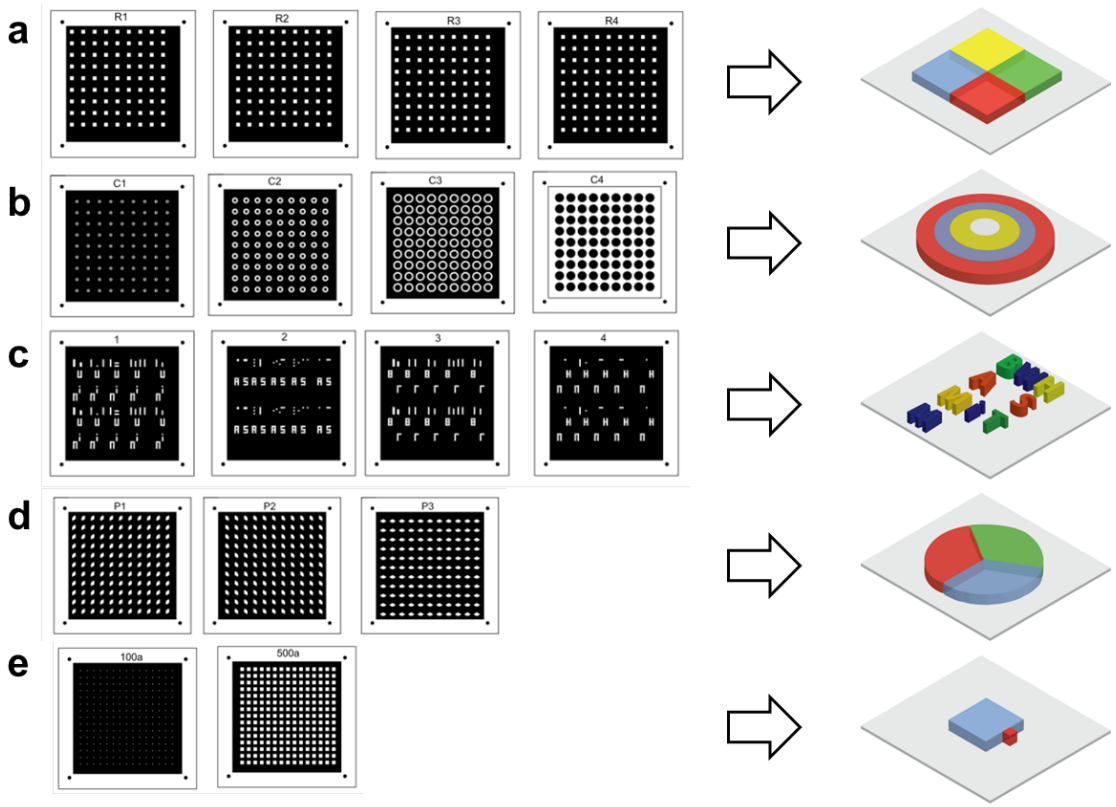

Figure s1: Masks were designed using computer aided design software to create digitally sculpted tissue constructs in a stepwise fashion. Steps and shapes were represented by the numbers printed on the masks (ii) (A) 4 masks were created for the square geometry by shifting each mask by 500 μm in up, down and side orientation. (B) 4 masks were created for the concentric circle geometry with diameters 200 μm, 400 μm, 800 μm, and 1200 μm. The fourth mask was designed to surround the three concentric circles and represent the surrounding tissue. (C) 4 masks were designed to create a micro-scale printed script (BWH, BAMM, HST, and MIT). The width and height of a letter within the script were 600 μm and 1000 μm respectively. (D) 3 masks were designed to create 120° sections of a pie chart with a diameter of 1000 μm. (E) A 100 μm by 100 μm square mask and a 500 μm by 500 μm square mask were aligned to have tangential contact.

To microfabricate neuron encapsulating tissue prototypes, three kinds of photomasks were designed with arrays of 100 × 100 μm, 200 × 200 μm and 500 × 500 μm squares. The

schematic illustration of this fabrication process is shown in **Fig. 1**. We used a spacer height of 150 µm, resulting in elements with dimensions of 100 × 100 × 150 µm, 200 × 200 × 150 µm and 500 × 500× 150 µm (**Fig. 2a**). To fabricate neuron encapsulating elements, 50 µL of neuron-suspended hydrogel (GelMA) precursor solution (*i.e.* 5 % (w/v) GelMA and 0.5 % (w/v) Irgacure 2959 photoinitiator) with 5×10^6 cells/mL was pipetted onto a polystyrene substrate. A TMSPMA modified glass slide was used (**Fig. s2e**). The photomask was then placed on the TMSPMA glass slide and exposed to UV light (**Fig. s2e**). The cover slide was washed with PBS to remove excess precursor solution. At the end of the microfabrication process, the constructs were placed in a 6-well plate for culture. Two photocrosslinking parameters (intensity and exposure duration) were optimized to fabricate neuron encapsulating tissue constructs (**Fig. 3k&l**). To assess UV intensity effect on cell viability, we used 6.9 mW/cm^2 and 2.9 mW/cm^2 intensities at UV exposure duration of 20 seconds. We also tested three different exposure durations of 20, 30, and 60 seconds (at an intensity of 2.9 mW/cm^2). After crosslinking and PBS washing steps, neuron encapsulating elements were stained with a live/dead assay. We have also employed the digitally specified tissue prototype fabrication to engineered a neuron cell encapsulating tissue composed of a 100 × 100 × 150 µm and a 500 × 500× 150 µm element (**Fig. 2s-y**).

The process to fabricate single-layer multi-element hydrogels was repeated to fabricate 3D multilayer prototypes via a layer-by-layer approach mimicking that in the semiconductor processing to build multilayer structures. Multiple glass slide layers (150 µm thick for each layer) were added in the base part to achieve the desired height and thickness for the multiple layers. For instance, the first layer of tissue prototypes was fabricated with a 150 µm thick base, and in order to fabricate the second layer, another glass slide layer of 150 µm was placed on top of the previous, giving the second layer a thickness of 300 µm in total (**Fig. 1**). After crosslinking, uncross-linked hydrogel precursor solution was washed away with PBS.

Another gel solution was then pipetted and crosslinked. The second layer was fabricated on the top of the first layer with a 300 µm thick base, while the third layer was fabricated on the top of the second layer with a 450 µm thick base. Then, we obtained three-layer tissue prototypes with 150 µm thick layers. To better observe and image the multiple layers, the glass slide layers of thickness 150µm were doubled for each layer giving them each a thickness of 300µm.

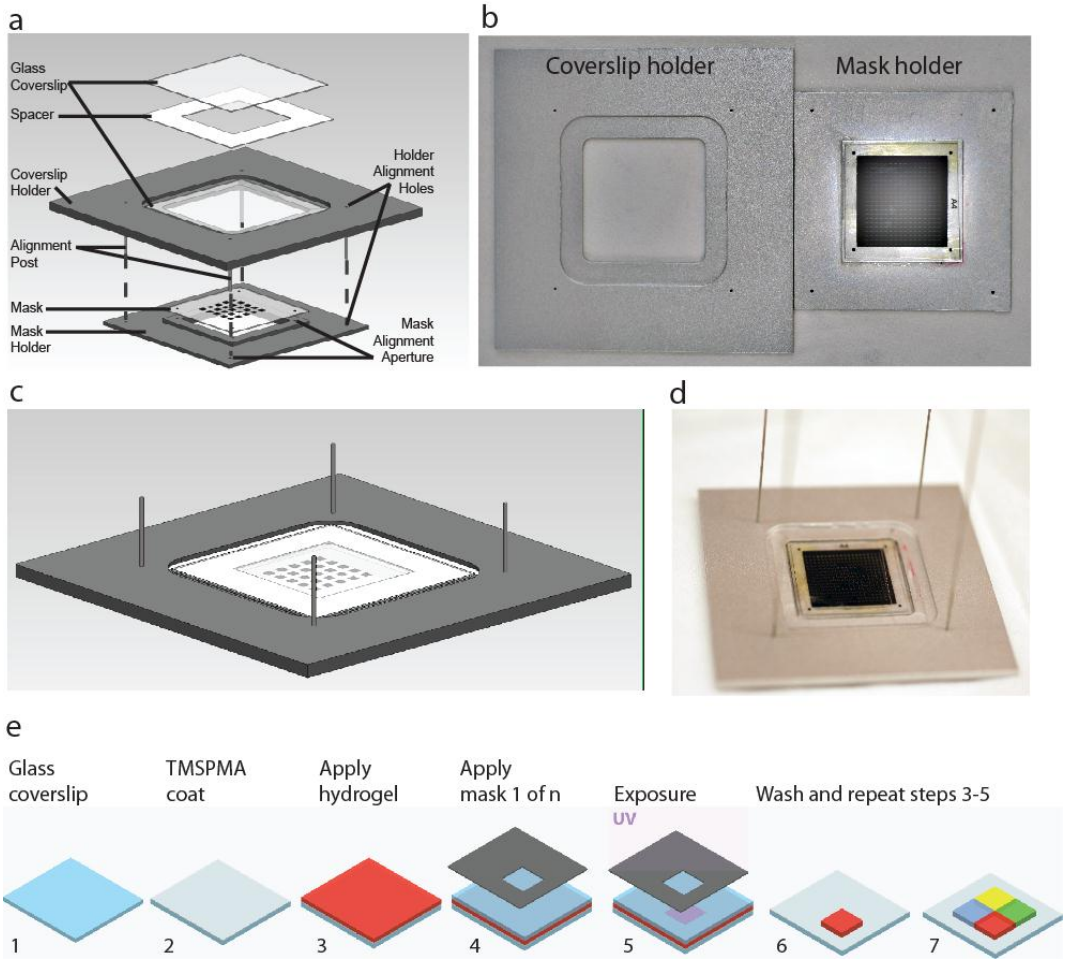

Figure s2: Mask alignment setup and protocol for digitally specified tissue prototypes

The mask alignment and digital sculpting device is composed of seven components (**Fig. s2**): fabrication compartment composed of a glass cover slip (i), spacer to control the thickness of each layer (ii), a treated glass cover slip on which the sculpted hydrogels are immobilized (iii),

fabrication chamber (iv) with alignment pins (v), the predesigned mask (vi) and a mask holder (vii) which aligns with the help of the pins on the chamber.

The precision of the alignment during fabrication was analyzed using NIH ImageJ program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/). Region of interests (ROIs) were selected and analyzed using 4x microscope images (**Fig. s3a**) for square, radially fractioned circular (radial circular) and concentric circular geometries. The centroids of individual building blocks were determined using the centroid function in ImageJ. The centroid of the first unit was used as the reference point in determining the alignment precision of the remaining units based on the computer aided design coordinates (**Tables s2-s4**). The differences in X and Y axes and the total distance between the centroids of the building blocks were measured in reference to the first crosslinked unit's centroid (**Fig. s3b**). The alignment precision was reported in X, Y axes and in total distance between the centroids (**Fig. s3c**). The method achieved high alignment precision in digital sculpting tissue prototypes: 10.9 μm (±8.2 μm) for square geometry, 11.2 μm (±4.5 μm) for radial circular geometry, and 27.8 μm (±9.8 μm) for concentric circular geometry (mean ± STD, n = 16-24 measurements for each geometry). As the alignment precision was analyzed and presented in all directions for multiple samples, the same alignment precision would hold across layers as also displayed in Figure 4 for three different geometries since the same set of masks with the same alignment pins are used. We did not observe a change in alignment precision across layers within the 3 layers that were fabricated.

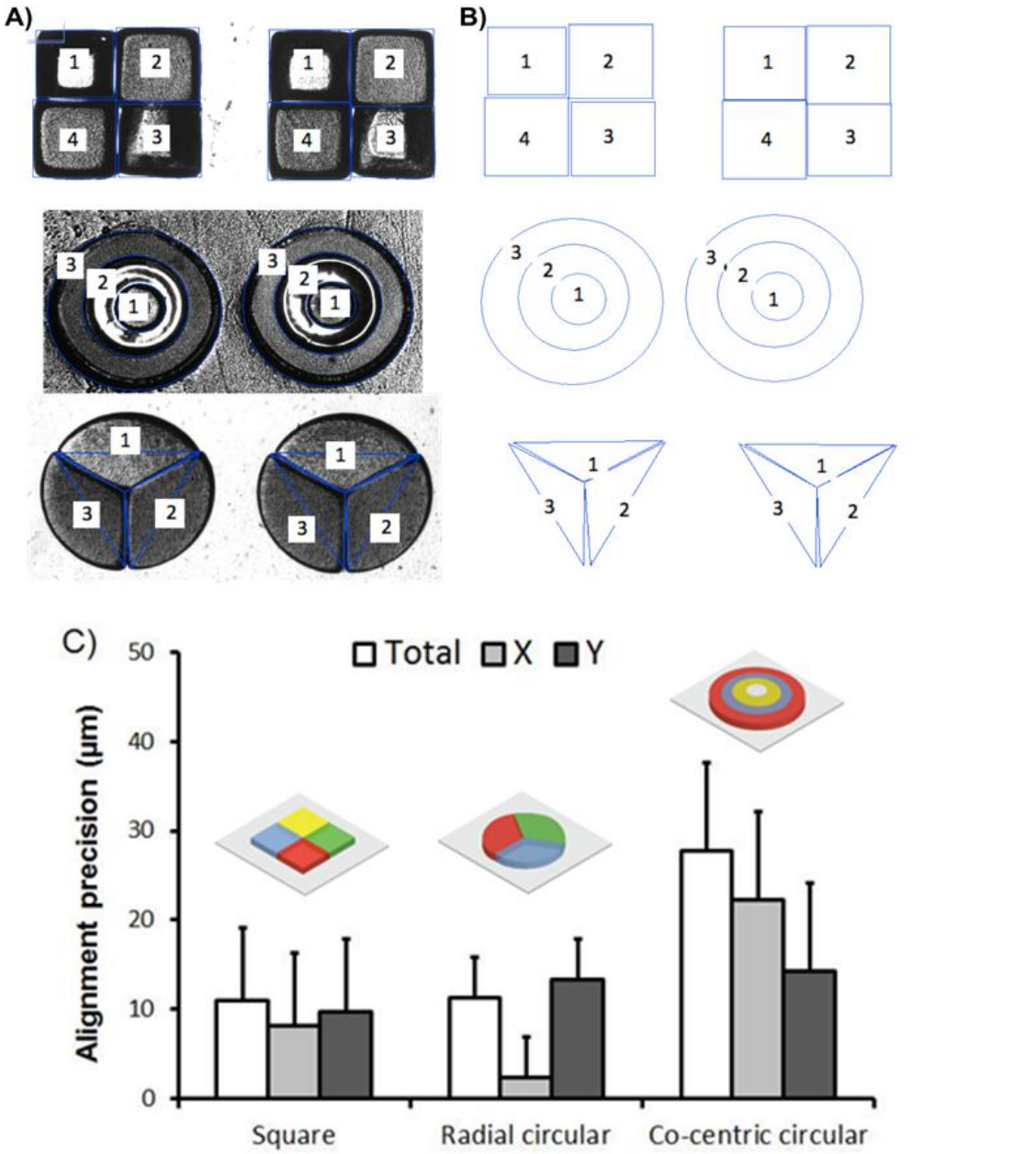

Figure s3: Quantification of alignment precision for square, radial circular, and concentric circular geometry tissue prototypes. a) Typical microscope images, and b) alignment markers used for quantification. c) Quantified alignment precision for tissue prototypes in X, Y directions and the total distance. The method achieved high alignment precision in digital sculpting tissue prototypes: 10.9 µm (±8.2 µm) for square geometry, 11.2 µm (±4.5 µm) for radial circular geometry, and 27.8 µm (±9.8 µm) for concentric circular geometry (mean ± standard deviation, n = 16-24 measurements each). Error bars in the figure represent mean ± standard deviation of the mean.

Gelatin (Type A, 300 bloom from porcine skin), methacrylic anhydride (MA) and 3-(trimethoxysilyl) propyl methacrylate (TMSPMA) were purchased from Sigma-Aldrich (St. Louis, MO). Photoinitiator 2-hydroxy-1-[4-(hydroxyethoxy)-phenyl]-2-methyl-lpropanone (Irgacure 2959) was purchased from Ciba Geigy (Igracure^{TM} 2959, Ciba Speciality

Chemicals, Tarrytown, NY, USA). The live/dead viability/cytotoxicity kit for mammalian cells was purchased from Invitrogen Corporation (Molecular Probes, Invitrogen, Carlsbad, USA). Neurobasal™, MEM and B27 supplement were purchased from Invitrogen Corp. (Carlsbad, CA). Glass slides and coverslips (VWR VistaVision™ microscope cover glasses, 25 × 25 mm) were purchased from Fisher Scientific (Philadelphia, USA).The Omnicure® S2000 UV/Visible Spot Curing System from EXPO Photonic Inc. (Ontario, CANADA) was used to polymerize hydrogels. We used the following antibodies: mouse monoclonal anti-Tau-1 (Millipore Bioscience Research Reagents, Darmstadt, GERMANY), rabbit polyclonal anti-GFAP (1:500 dilution; DakoCytomation), mouse anti-GAD65 (Millipore Bioscience Research Reagents, USA), rabbit anti-CaMKII (Santa Cruz Biotechnology, USA); Alexa Fluor® 488 goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) and Alexa Fluor® 568 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) were used as secondary antibody. The fabricated tissue prototypes were stained with Procion MX dyes (Sigma-Aldrich, St. Louis, MO, USA) in four different colors: red, blue, green, and yellow.

Gelatin methacrylate (GelMA) was dissolved at 10 % (w/v) gelatin (Type A, 300 bloom from porcine skin) in Dulbecco's phosphate buffered saline (DPBS; GIBCO) at 50 ºC for 1 hour. Methacrylic anhydride (94 %, MA, Sigma-Aldrich, St. Louis, MO) was added at 50 ºC under stirring condition for 2 hours. The solution was dialyzed in distilled water using a 12-14 kDa dialysis bag (Fisher Scientific, Philadelphia, USA) for 1 week at 40 ºC. The dialyzed solution was then transferred into 50 mL centrifuge tubes and lyophilized for one week to synthesize usable GelMA in powder form. GelMA precursor solution was prepared by mixing 5% (wt/wt) gelatin methacrylate and 1% (wt/wt) 2-hydroxy-1-(4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone photo-initiating powder (Igracure™ 2959, Ciba Speciality Chemicals, Tarrytown, NY, USA) in DPBS with or withour cells.

In accordance with institutional guidelines for care and use of animals, dissociated cortical neurons were isolated. Briefly, neural cortex was extracted from postnatal 1 day old (PD 1) Sprague Dawley® rats (Charles River Labs, MA, USA). Cortex tissue was dissociated by trituration after digestion using papain (20 U/ml). The cells were then suspended in neurobasal medium with B27 supplement and 500 µM glutamine. The medium for new cultures was supplemented with 25 µM glutamate and 25 µM ß-mercaptoethanol. Cultured neurons were then suspended in GelMA precursor solution. Glass cover-slips with neuron-encapsulated tissue elements were placed in a six-well plate, and supplemented with 3 mL neural culture medium. Culture conditions were maintained at 37 ºC with a 95% relative humidity, and 5 % CO_2. Media was changed biweekly by replacing half with fresh media. To assess the viability of neurons in tissue constructs, live/dead assay (Molecular Probes, Invitrogen, Carlsbad, CA, USA) were conducted over time. Neuron encapsulating elements were incubated with live/dead staining solution that was composed of 4 µL ethidium homodimer-1 and 1 µL calcein-AM. Fluorescent images were taken (Nikon T2000) and NIH ImageJ software was used to analyze the images and quantify the results. Cell viability assay was conducted on days 0, 3 and 6. Long-term culture characterization included the neurite growth analysis **(Supplementary Video)** and functional immunostaining of the encapsulated neurons in 3D elements at the end of a 3-week culture period.

Functional immunofluorescent staining to identify cellular composition in digitally specified neural tissue constructs. The glass slides with neuron cell encapsulating elements were fixed with 4 % paraformaldehyde and permeabilized for 30 min with 0.5 % Triton X-100 and then blocked with 10 % goat serum albumin in phosphate buffered solution. These glass slides were washed with PBS and incubated separately with mouse monoclonal anti-Tau-1 / rabbit polyclonal anti-GFAP, mouse anti-GAD65 / rabbit anti-CaMKII, or mouse monoclonal anti-Tau-1 / rabbit anti-CaMKII, overnight at 4 ºC. After washing with PBS, glass slides were

incubated overnight at 4 ºC with Alexa Fluor® 488 goat anti-mouse IgG / Alexa Fluor® 568 goat anti-rabbit IgG secondary antibodies, as well as DAPI (Invitrogen, Carlsbad, CA, USA). The slides were transferred onto 24 × 60 mm glass slides and mounted using SlowFade® Gold antifade reagent (Invitrogen, Carlsbad, CA, USA) and scanned with a confocal microscope and imaged. The z-stacks were then merged using NIH ImageJ, and a video **(Supplementary Video)** showing 3D neural network cultured in elements was made with Imaris software (Bitplane Inc., South Windsor, CT).

Cell encapsulation and tracking in digitally specified tissue constructs. Three cell types were used: embryonic stem cells (ESCs), human umbilical vein endothelial cells (HUVECs), and NIH 3T3 fibroblasts. ESCs were cultured in Glasgow Minimum Essential Medium (Sigma Aldrich, USA) supplemented with 15% Fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1% Penicillin Streptomycin (Invitrogen, Carlsbad, CA, USA) 1% Sodium Pyruvate (Invitrogen, Carlsbad, CA, USA) , 1% Non-essential amino acids (NEAA) (Invitrogen, Carlsbad, CA, USA), 0.1% B-mercaptoethanol (Invitrogen, Carlsbad, CA, USA) and 0.01% Leukemia Inhibitory Factor (MilliporeDarmstadt, Germany). HUVECs were cultured in Endothelial Cell Medium (Sigma). 3T3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% Fetal bovine Serum (FBS, Invitrogen) and 1% Penicillin Streptomycin (Invitrogen, Carlsbad, CA, USA). ESC, HUVEC and 3T3 cells were trypsinized and resuspended in hydrogel precursor solution (1:1 for cell medium suspension and GelMA solution) with a final concentration of $1x10^6$ cells/ml. For cell tracking test, cells were incubated with ER-Tracker™ 30 minutes before being resupended in hydrogel precursor solution: Red (BODIPY® TR glibenclamide, Invitrogen) for HUVEC, ER-Tracker™ Green (BODIPY® FL glibenclamide, Invitrogen) for ESCs, and ER-Tracker™ Blue-White DPX (Invitrogen, Carlsbad, CA, USA) for 3T3 cells. The multiple cell type encapsulating multi-element tissue prototypes were then fabricated following the protocol

described above. Florescent images of the tissue prototypes labeled with cell tracker were taken using an inverted microscope (Nikon TE2000).

Assessing cell viability in long-term culture of digitally specified tissue prototypes. Cell viability was tested using a live/dead assay at day 0, day 2, and day 3. Tissue prototypes for cell live/dead assay were prepared using cells without cell tracker stains. Tissue prototypes were first washed with DPBS and gels were incubated with 0.5 μl/ml of calcein-AM (for live cells) and 2 μl/ml of ethidium bromide (for dead cells) in DPBS. The fluorescent images were taken using an inverted microscope (Nikon TE2000). Cell viability was calculated as the average value of the fraction of live cells to the total number of cells taken from 5 samples. Viability of three different cell types encapsulated at varying concentrations, ESCs ($5x10^5$ cells/ml), HUVEC ($7.5x10^5$ cells/ml) and 3T3 ($1x10^6$ cells/ml), was quantified in fluorescent microscope images using the NIH ImageJ program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/). We cultured the prototypes with a mixed medium (1:1:1 ratio of ESC: HUVEC: 3T3 media) for a period of up to 3 days. Cell culture medium was changed daily.

Coloring of hydrogels. A single batch of 40% PEG solution with a 1% photo initiator concentration (Igracure^{TM} 2959, Ciba Speciality Chemicals, Tarrytown, NY, USA) was prepared which was then divided into equal volumes and colored with Procion MX Dyes (Sigma-Aldrich, St. Louis, MO, USA). Two factors were taken into consideration when determining dye percentages: desired color intensity and the crosslinking quality of the colored gel. An optimization among the two gave lead to the following dye percentages in the hydrogel solution (**Table s1**).

Statistical analysis. The experimental results on the effects of UV intensity (n=3), photocrosslinking time (n=3-8), and culture duration (n=3-8) on cell viability; and the effect of element size and culture duration on axon length (n=3-6) were analyzed by performing

analysis of variance (ANOVA) with Tukey's posthoc test for multiple comparisons. The statistical comparisons between the neuron and glial cells (n=4), between the excitatory and inhibitory cells (n=5) were performed by non-parametric Mann-Whitney U test. Statistical significance threshold was set at 0.05 ($p<0.05$) for all analyses. Data are presented as mean ± standard deviation (STD) of the mean.

Table s1: Percentages of the dyes added to the PEG hydrogel solution and their relative cross-linking times

Color	%	Crosslink (sec)
Red	2.5	28
Blue	1.2	29
Green	1.9	29
Yellow	2.17	25
Orange	2.33	26.5

Table s2: Designed distances between centers of geometries according to a reference point of the first-crosslinked geometry's center

Number of Geometries (Fig S4 B)	Squares	Concentric Circular	Radial Circular
Difference between 1-2	500 μm	0 μm	288.7 μm
Difference between 1-3	707.1 μm	0 μm	288.7 μm
Difference between 1-4	500 μm	-	-

Table s3: Designed differences in X axis according to a reference point of the first-crosslinked geometry's center

Number of Geometries (Fig S4 B)	Squares	Concentric Circular	Radial Circular
Difference between 1-2	+500 μm	0 μm	+144.3 μm
Difference between 1-3	+500 μm	0 μm	-144.3 μm
Difference between 1-4	0 μm	-	-

Table s4: Designed differences in Y axis according to a reference point of the first-crosslinked geometry's center

Number of Geometries (Fig S4 B)	Squares	Concentric Circular	Radial Circular
Difference between 1-2	0 μm	0 μm	-250 μm
Difference between 1-3	-500 μm	0 μm	-250 μm
Difference between 1-4	-500 μm	-	-

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