Supporting Information

Experimental Section

Materials. Gelatin (Type A, from porcine skin), sorbitan monooleate (Span® 80), toluene (99.8 %), sodium alginate and calcium chloride were all obtained from Sigma-Aldrich (St. Louis, MO) and used as received to produce uniform gelatin microspheres with a fluidic device. Poly(D,L-lactide-*co*-glycolide) (lactide/glycolide=75/25, M_w≈66,000-107,000, Sigma-Aldrich) was employed to fabricate the inverse opal scaffolds. The water used in all experiments was obtained by filtering through a set of Millipore cartridges (Epure, Dubuque, IA).

Preparation of inverse opal scaffolds. We fabricated the uniform microspheres of gelatin and inverse opal scaffolds of PLGA by following our recently published procedures.[1,2] Briefly, a methanol suspension of gelatin microspheres (200 or 300 μm in diameter, ~1.5 wt%) was placed in a 50-mL centrifuge tube, capped, and sonicated for 10 s. The wall of the centrifuge tube was then gently tapped to obtain a cubic closest packed (ccp) lattice. While immersed in methanol, the tube was capped and placed in an oven heated at 65 °C for 1 h to form necks between the adjacent gelatin microspheres. After cooling down to room temperature for 30 min, the ccp lattice pellet was harvested by a spatula, placed on a filter paper to evaporate methanol, and then infiltrated with a PLGA solution in 1,4-dioxane (20 wt%). After removing the excess PLGA solution with filter paper, the lattice pellet containing PLGA solution was frozen in a refrigerator (-20 °C) for 5 h, and then lyophilized overnight in a freeze-dryer (Labconco Co., Kansas City, MO). The pellet with freeze-dried PLGA was immersed in ethanol for 5 min under vacuum to eliminate air bubbles trapped inside and subsequently transferred to 900 mL of water heated at 45 °C for 3 h to remove the gelatin microspheres under magnetic stirring.

Cell culture and seeding. NIH/3T3 mouse fibroblasts (CRL-1658), MG63 human osteoblasts (CRL-1427), HepG2 human hepatoma cells (HB-8065), SVEC4-10 mouse endothelial cells (CRL-2181), SK-BR-3 human breast cancer cells (HTB-30), and U-87 MG human brain glioblastoma cells (HTB-14) were all obtained from American Type Cell Culture (ATCC). The cells were maintained in the corresponding culture media according to vendor's instructions at 37 °C and 5% CO_2, and passaged weekly. Rat bone marrow stromal cells were obtained from Dr. Thomopoulos at Washington University School of Medicine. The cells were maintained in

Minimum Essential Medium α (MEM-α, Invitrogen) supplemented with 20% heat-inactivated fetal bovine serum (FBS, ATCC) and 1% penicillin-streptavidin (Invitrogen) at 37 °C and 5% CO_2, and passaged weekly.

Prior to cell seeding, cover glasses (Thermo Fisher Scientific, Waltham, MA) and scaffolds were sterilized with 70% ethanol for 2 h and then UV irradiation overnight, washed with PBS (Invitrogen, Carlsbad, CA) three times and stored in culture medium. In the case of cover glasses, 24-well plates were used, and cells were seeded at a density of 5×10^4/well. The cells were incubated for 12 h before MTT staining. In the case of scaffolds, about 2×10^5 cells were used to seed into each scaffold using a spinner flask (125 mL capacity, Proculture™, Corning, Corning, NY) at 65 rpm for 2 h. The cell-seeded scaffolds were then transferred into a 12-well plate (one per well) and cultured in the culture medium. Dulbecco's modified eagle medium (DMEM; Invitrogen) free of phenol red was used to reduce possible signal interference during PAM imaging. All cultures were kept in an incubator at 37 °C in a humidified atmosphere containing 5% CO_2 and the media were changed every other day.

For cell invasion experiment, monolayers of fibroblasts were formed in each well of a 24-well plate. The scaffolds were carefully placed in the wells (one per well) on top of the cell monolayers. The culture was kept in still, and culture media were not changed during the entire 7-day culture period.

The protocol for mESCs culture was identical to what was described previously.[3] RW4 mouse ES cells were obtained from Dr. Gottlieb at WUSM and cultured in culture flasks coated with a 0.1% gelatin solution. 1000 U/mL leukemia inhibitory factor (LIF, Chemicon, Temecula, CA) and 10^{-4} M β-mercaptoethanol (BME, Invitrogen) were added to maintain their undifferentiated state. Cells were cultured in complete media consisting of DMEM supplemented with 10% new born calf serum, 10% fetal bovine serum (Invitrogen), and 0.3 mM of each of the following nucleosides: adenosine, guanosine, cytosine, thymidine, and uridine (Sigma-Aldrich), and passaged every two days. Undifferentiated mESCs were induced to form EBs containing neuron precursor cells using the 4-/4+ retinoic acid treatment protocol.[4] ESCs were cultured in 100 mm Petri dishes coated with a 0.1% agar solution (Sigma-Aldrich) in complete medium in the absence of LIF and BME for 4 days. Retinoic acid (Sigma-Aldrich) at 500 n M was then added to the complete media for the final 4 days of culture. Media were changed every other day during the 8-day process.

Multicellular tumor spheroids were induced to form by seeding 10^4 SK-BR-3 cells into wells of a 96-well plate pre-coated with methylcellulose (Sigma-Aldrich) hydrogels.[5]

MTT formazan and f-actin staining. Cells were incubated with 5 mg/mL MTT solution in PBS (40 µL/well in culture medium to a final concentration of 0.05 w/v%) for 3 h at 37 °C. This concentration and the incubation parameters were used in all experiments unless otherwise specified. When f-actin staining was needed, the cells were briefly fixed with 3.7% formaldehyde for 5 min, washed with PBS and further incubated with Alexa 568-phalloidin (Invitrogen) for 20 min at RT, followed by another PBS washing.

Photoacoustic microscopy and signal processing. The integrated photoacoustic and fluorescence confocal microscopy (FCM) system used a tunable dye laser (pulse duration: 7 ns) as the irradiation source.[6] The samples were immobilized on the bottom of a petri dish filled with PBS. The laser beam was focused onto the sample by an objective lens (NA: 0.2; magnification: 13.3). The generated photoacoustic waves were detected using a 75-MHz ultrasonic transducer (V2022 BC, Olympus NDT). The FCM subsystem shared the same laser source and objective lens. A dichroic mirror (DMLP605, Thorlabs, Newton, NJ) and an emission filter (FF01-624/40-25, Semrock, Rochester, NY) allowed transmission of fluorescence (above 605 nm) emitted from the sample. The fluorescence light passing through the pinhole (pinhole diameter, 50 µm) was collected by a photomultiplier (H6780-20, Hamamatsu, Bridgewater, NJ). The photoacoustic signals and fluorescence signals were acquired through a data acquisition card (CS 14200, Gage Applied, Lockport, IL). The automatically co-registered photoacoustic and fluorescence images were acquired simultaneously by 2-D raster scanning of the sample along the transverse plane.

Scaffolds with stained cells were removed from culture medium, placed in a PDMS mold with PBS, and imaged with photoacoustic microscopy. The PA waves were focused and detected using a 50-MHz ultrasonic transducer (V214-BB-RM, Olympus NDT, Kennewick, WA). Through time-resolved ultrasonic detection at each location, 1-D depth-resolved image (A-scan) was obtained. A 1D scan along the x direction acquired a 2D B-scan image. Thus, 2D raster scanning along the transverse plane produced complete volumetric information. Each A-scan was acquired within 2 µs, 1D scan for a single B-scan image took ~0.5 s, and a 2D bi-directional scan over a 6 mm by 6 mm area, which covered the whole scaffold sample, took ~5 min.

High-resolution PAM was performed on a system described as follows.[7] Prior to imaging,

the samples were briefly fixed with 3.7% formaldehyde in PBS, placed on a glass slide, and covered with ultrasound gel to couple with a water tank. The laser pulses with a wavelength of 532 nm were focused by an optical objective to irradiate the sample. A 40-MHz ultrasonic transducer detected the resulting PA signals in the transmission mode. By mechanically scanning the objective and the transducer in raster mode along the transverse plane and projecting the maximum amplitude of the volume onto the scanning plane, an image of the sample could be obtained. The image resolution, as determined by the numerical aperture (0.6) of the objective, was about 0.4 μm. Absorption contrast with a relative sensitivity of 100% was shown in the image.

All PAM and FCM data were analyzed by user-defined MATLAB (MathWorks, Natick, MA) programs.

MTT assay. After PAM imaging of the scaffolds, the samples were collected in a 12-well plate (one scaffold per well). 1 mL 1-propanol was added to each well to completely dissolve the MTT formazan crystals inside each sample. Optical density was measured at 562 nm using a spectrophotometer (Infinite 200, TECAN).

Scanning electron microscopy. Scanning electron microscopy (Nova NanoSEM 2300, FEI) was used to characterize PLGA inverse opal scaffolds. Prior to imaging, the scaffolds were sputter-coated with gold for 60 s. Images were taken at an accelerating voltage of 5 kV.

References

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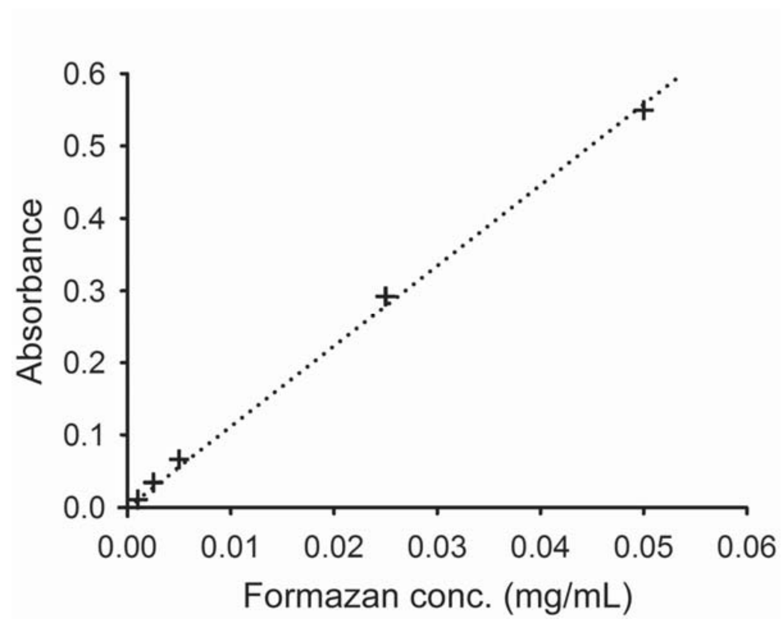

***Figure S1*.** The calibration curve of MTT formazan dissolved in 1-propanol, as measured using a spectrophotometer. The measurement was carried out in a 96-well plate, and 150 μL of the solution was added to each well.

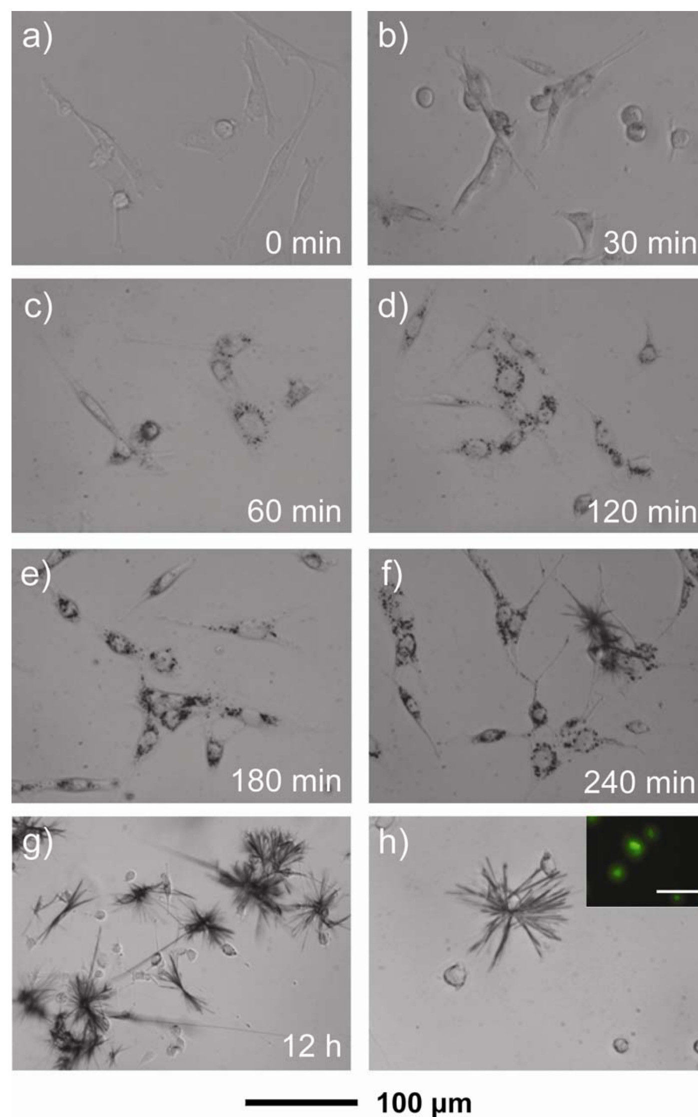

Figure S2**.** The metabolism of MTT as a function of time. a-f) Cellular accumulation of MTT formazan as the incubation time was increased. The fibroblasts incubated with MTT for more than 4 h began to exocytose MTT formazan crystals. g) After 12 h of incubation, a large number of formazan crystals were exocytosed from the cells; and h) bright-field optical micrograph and the corresponding fluorescence micrograph (inset) after LIVE/DEAD staining of the sample in (g). The live and dead cells were stained green and red, respectively, to reveal that the cells in the process of exocytosis were still viable. Scale bar in the inset: 100 µm. In this case, the MTT was kept in the culture medium throughout the experiment.

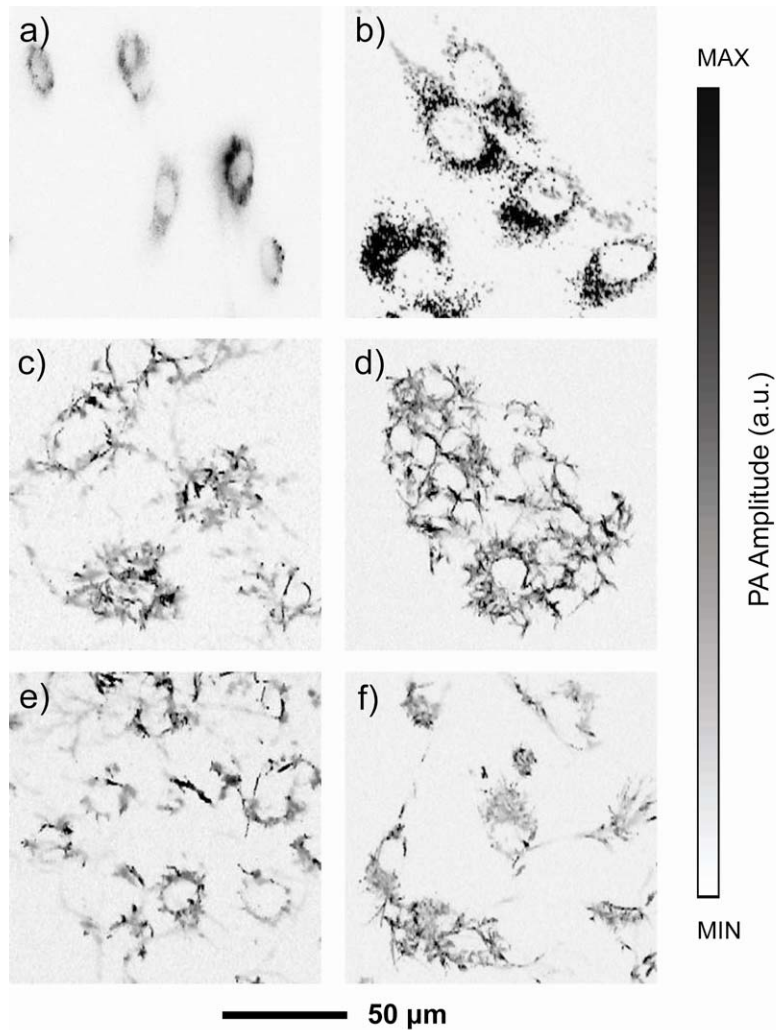

***Figure S3*.** High-resolution (0.4 μm lateral) PAM images of different types of cells obtained in conjunction with MTT staining. a) NIH/3T3 mouse fibroblasts; b) MG63 human osteoblasts; c) rat bone marrow stromal cells; d) a cluster of HepG2 human hepatoma cells; e) SVEC4-10 mouse endothelial cells; and f) U-87 MG human brain glioblastoma cells.

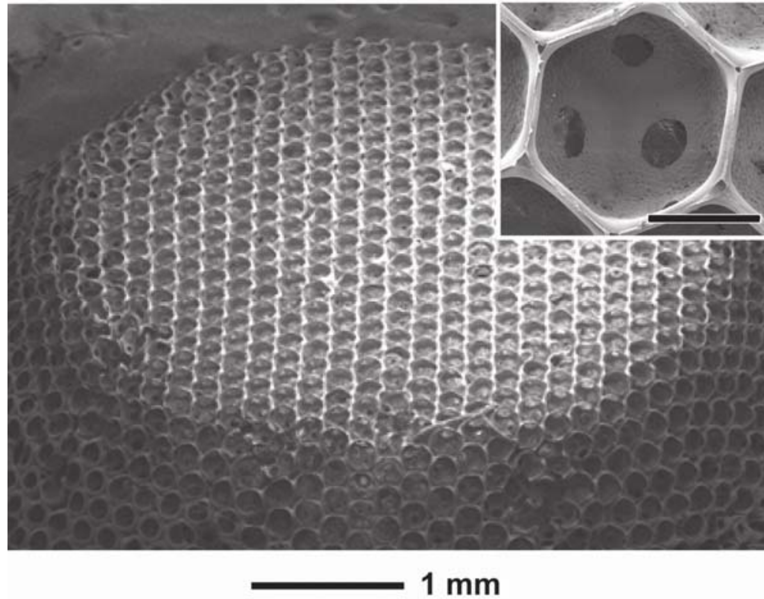

Figure S4. SEM images of a typical PLGA inverse opal scaffold. The inset shows an enlarged view of a pore, where three windows are visible. These windows interconnect the adjacent pores, facilitating the transport of nutrients, oxygen, and wastes, as well as the migration of cells. Scale bar in the inset: 100 μm.