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Supporting Information

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Optical-Resolution Photoacoustic Microscopy for Volumetric and Spectral Analysis of Histological and Immunochemical Samples**

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Experimental

Materials. Gelatin (type A, from porcine skin), sorbitan monooleate (Span® 80), and toluene (99.8%) were obtained from Sigma-Aldrich (St. Louis, MO) and used as-received for fabricating uniform gelatin microspheres. PLGA (lactide/glycolide=75:25, MW≈66,000 to 107,000) was also obtained from Sigma-Aldrich and used as-received for fabricating the inverse opal scaffolds. The water used in all experiments was filtered through a set of Millipore cartridges (Milli-Q, Billerica, MA). Chemicals used for staining cells were all obtained from Sigma-Aldrich unless otherwise noted.

Fabrication of inverse opal scaffolds. The PLGA inverse opal scaffolds were fabricated by modifying a previously reported protocol.[6d,7e,7i,8h,9,11] Briefly, uniform microspheres of gelatin were fabricated and dispersed in methanol (*ca.* 1.5 wt%) in a 50-mL centrifuge tube, and the tube was gently tapped to crystalize the microspheres into a cubic-closed packed (ccp) lattice. The resultant ccp lattice was placed in an oven at 65 °C for 1 h to induce necking between adjacent microspheres. After cooling to room temperature, the lattice was carefully harvested using a spatula, placed on a filter paper to remove methanol, and then infiltrated with a PLGA solution in 1,4-dioxane (18 wt%). After removing the excess PLGA solution with a filter paper, the pellet containing PLGA solution was frozen in a refrigerator (-20 °C) for 5 h, and then lyophilized in a freeze-dryer (Labconco, Kansas City, KS) overnight. The sample was placed in ethanol, briefly vacuumed for 5 min to eliminate air bubbles, and placed in 900 mL of water heated at 45 °C under gentle stirring for 4 h to dissolve the gelatin microspheres. All the scaffolds were sterilized in 70% ethanol overnight prior to cell culture.

Cell culture. MC3T3-E1 mouse pre-osteoblasts and SK-BR-3 human adenocarcinoma (breast tumor) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained in Minimum Essential Medium Alpha (for MC3T3-E1, Invitrogen, Carlsbad, CA) or McCoy's 5A Medium Modified (for SK-BR-3, ATCC) supplemented with 10% fetal bovine serum (FBS, ATCC) and 1% penicillin-streptomycin (P/S, Invitrogen) in an incubator. The culture medium was replaced every other day. Roughly 5×10^5 cells (MC3T3:SK-BR-3 = 10:1 in number) were used to seed each scaffold, using a spinner flask at 70 rpm for 2–3 h. The scaffolds were transferred into the wells (one scaffold per well) of a 12-well plate (non-tissue culture treated) and rinsed with phosphate-buffered saline (PBS, Invitrogen), followed by

the addition of 1 mL culture medium into each well.

Histology and immunochemistry. The histology section was kindly provided by the Elvie L. Taylor Histology Core Facility at Washington University School of Medicine. The section was de-paraffinized in two exchanges of xylene and rehydrated in a graded ethanol series (50% through 0% in water). The sample then went through a standard Masson's trichrome staining procedure. For immunostaining of f-actin, the cells were first incubated in a streptavidin-biotin blocking solution (Vector Laboratories, Burlingame, CA) for 15 min, and then permeabilized in PBS containing 1.5% bovine serum albumin (BSA) and 0.1% Triton X-100. The cells were incubated in sequence with biotin-phalloidin (1:20 dilution, Biotium, Hayward, CA) for 30 min, streptavidin-HRP (1:100 dilution, Invitrogen) for 1 h, and ImmPACT DAB peroxidase substrate (Vector Laboratories) for 2–5 min. The cells were rinsed with water three times between adjacent sessions of incubation. For immunostaining of human epidermal growth factor receptor 2 (HER2) on the surfaces of SK-BR-3 cells, the cells were incubated in sequence with mouse anti-HER2 primary antibody (specific to human, 1:100 dilution, Invitrogen) overnight at 4 °C, HRP-goat anti-mouse IgG secondary antibody (1:200 dilution, Invitrogen) for 1 h, and VECTOR NovaRED peroxidase substrate (Vector Laboratories) for 2–5 min. The cells were rinsed with water three times between any adjacent incubation sessions.

Optical-resolution photoacoustic microscopy. The OR-PAM (Fig. S1) system used in this study had a lateral resolution of *ca.* 5 μm, an axial resolution of *ca.* 15 μm and a penetration depth of *ca.* 1.0 mm in soft tissues.[3] A tunable dye laser (CBR-D, Sirah, Kaarst, Germany) pumped by a Nd:YLF laser (INNOSAB, 523 nm, Edgewave, Würselen, Germany) was the excitation source. The short pulses (5 ns) from the dye laser were focused by a microscope objective (NA = 0.1, Olympus America, Center Valley, PA) into the sample surface. The PA signals were detected using a spherically focused ultrasonic transducer (V214-BC-RM, central frequency: 50 MHz, Olympus-NDT, Waltham, MA), which was placed confocally with the objective. An ultrasound/light combiner, composed of a thin layer of silicone oil sandwiched between a right-angle prism and a rhomboid prism, was used for the co-axial alignment of the optical and acoustic beams. A plano-convex lens on top of the combiner corrected the refraction of the prisms and water. Distilled water was used as the matching medium for acoustic propagation. A motion controller provided trigger signals for laser firing, data acquisition, and

raster scanning. While the lateral resolution of the system is defined by the diffraction-limited optical focus, the axial resolution is determined by the bandwidth of the acoustic detection.

For cell imaging where higher spatial resolution was required, we used a submicron OR-PAM with tighter optical focusing achieved via a water-immersion objective with an NA of 0.6 (Carl Zeiss, Thornwood, NY).[7l, 10] Compared with the system with a lateral resolution of 5 μm, the major differences included the laser and the PA detection. Here an integrated diode-pumped laser and optical parametric system (NT242-SH, Ekspla, Vilnius, Lithuania) was used. The pulse width was 5 ns, and the wavelength was tunable from 210 nm to 2600 nm. A focused ultrasonic transducer was placed directly in transmission mode, thus the ultrasound/light combiner was not required.

Image processing and spectral unmixing. The acquired 3D PA signals at each wavelength were first normalized by the laser fluence pixel-wise, and then Hilbert transformed to extract the amplitudes for further analysis. The spectral unmixing was then performed on either the 2D maximum-amplitude projection images, or the 3D volumetric datasets layer by layer, depending on the application. The unmixing method was similar to the quantification of the oxygen saturation of hemoglobin.[7a,7g] Briefly, from PA imaging at two or more wavelengths, the relative concentrations of different chromophores in the sample could be quantified through spectral analysis by solving linear equations, with their molar extinction coefficients as the prior information. Ultimately, these different chromophores (indicated by different colors) can be separated into individual components in 2D images or 3D volumes. All the data processing was performed using MATLAB R2010b (Mathworks, Natick, MA). The default algorithm of solving linear equations in MABLAB was singular value decomposition (SVD).

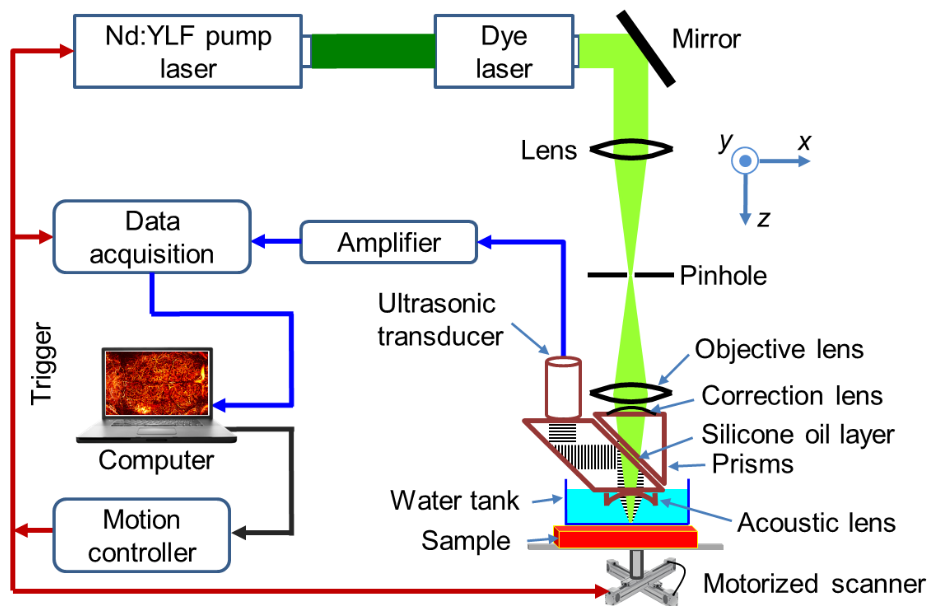


Figure S1. Schematic of the optical-resolution photoacoustic microscope (OR-PAM) used in the present work.

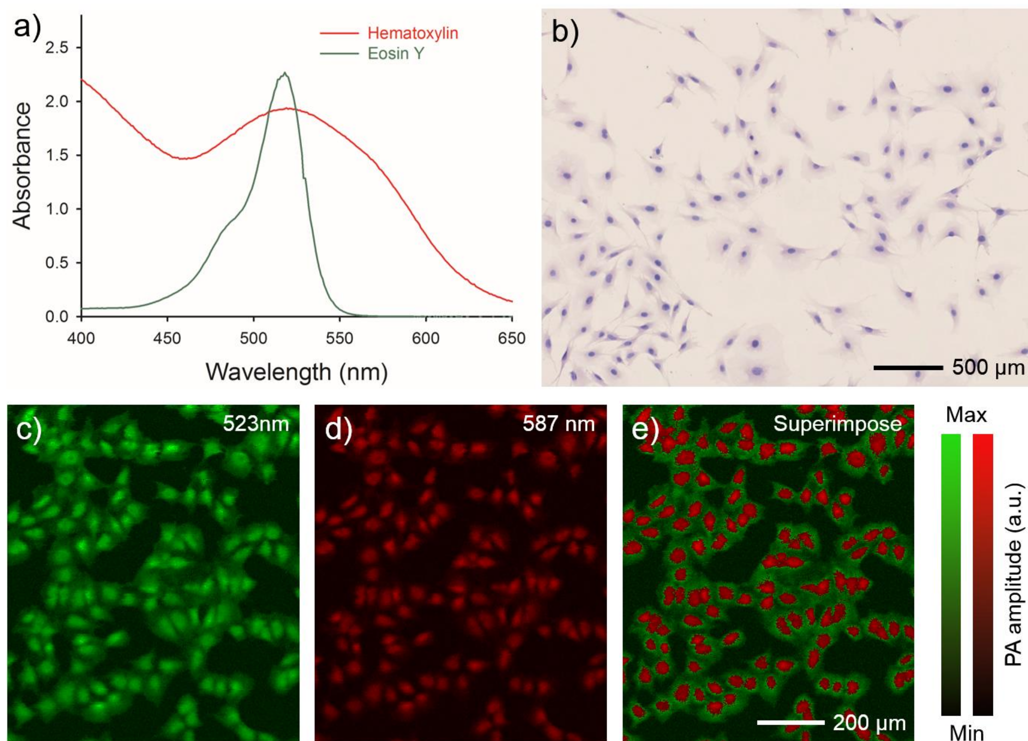
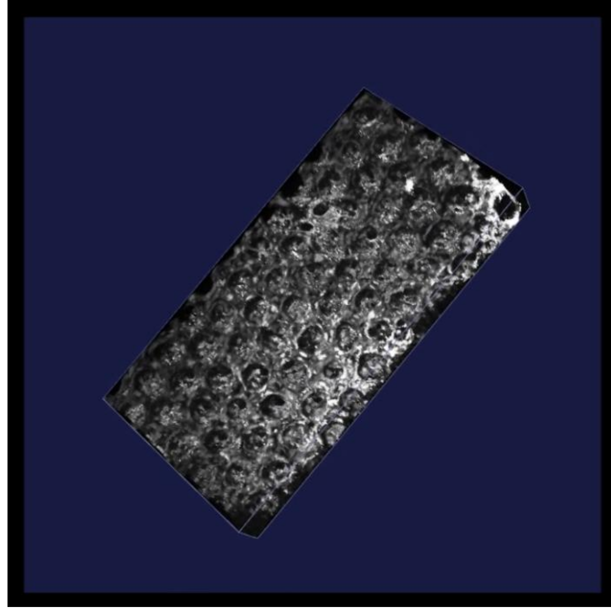
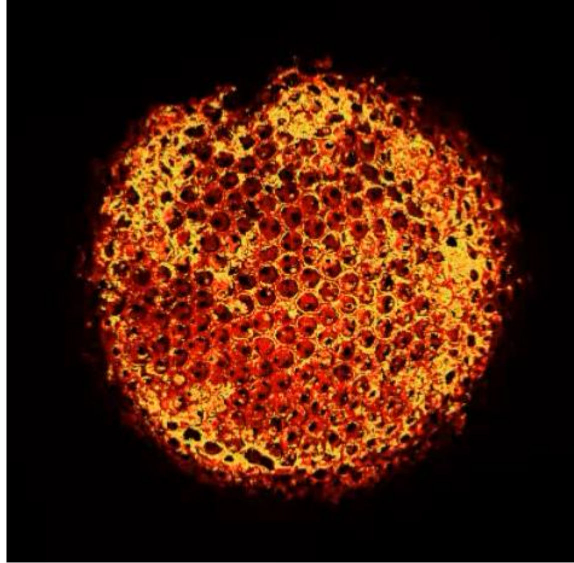


Figure S2. a) UV-vis spectra of hematoxylin and eosin (H&E). b) An optical micrograph of the MC3T3 pre-osteoblasts grown on a cover slip after H&E staining. c, d) OR-PAM MAP images of MC3T3 cells grown on a cover slip after H&E staining, acquired at wavelengths of 523 nm and 587 nm, respectively. The image at 587 nm showed a higher contrast of the hematoxylin-stained nuclei, whereas both components could be observed at 523 nm. e) A combined PAM MAP image showing the hematoxylin-stained nuclei (red) and eosin-stained cytoplasm (green). MAP stands for maximum amplitude projection.



Movie S1. Volumetric rendering of MC3T3 preosteoblasts cultured in a PLGA inverse opal scaffold for three days acquired using OR-PAM. The sample was stained for f-actin with biotin-phalloidin/streptavidin-HRP, followed by the DAB chromogenic development.



Movie S2. Volumetric rendering of MC3T3 pre-osteoblasts and SK-BR-3 breast tumor cells co-cultured in a PLGA inverse opal scaffold for 7 days. The video was acquired using OR-PAM. The sample was stained with biotin-phalloidin/streptavidin-HRP/DAB for f-actin, followed by staining with anti-HER2/HRP-secondary antibody/NovaRed for HER2 antigens expressed on the surfaces of SK-BR-3 cells. The SK-BR-3 cells were shown in red while the background MC3T3 cells were in brown.