SUPPLEMENTARY INFORMATION

Tunable Aggregation of Gold-Silica Janus Nanoparticles to Enable Contrast-Enhanced Multiwavelength Photoacoustic Imaging in Vivo

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Experimental Section

Materials. Unless otherwise specified, reagents were used as received without further purification. Gold(III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$ ≥99.9%), sodium citrate tribasic dehydrate (≥99%), 2-propanol (ACS reagent ≥99.5%), ethyl alcohol (ACS reagent ≥99.5%), ammonium hydroxide solution (ACS reagent 28.0-30% NH_3 basis), tetraethyl orthosilicate (TEOS, 99.999%), 4-mercaptobenzoic acid (4-MBA, 99%), poly(acrylic acid) solution (PAA, Mw: 250,000, 35 wt% in H_2O), Dulbecco's Phosphate Buffered Saline (DPBS, 10×, modified), gelatin from porcine skin (gel strength 300, Type A), and silica gel (pore size 60 Å, 230-400 mesh particle size, 40-60 mm particle size) were purchased from Sigma-Aldrich. Dulbecco's Modified Eagle Medium with 10% Fetal Bovine Serum (DMEM with 10% FBS, no phenol red) was purchased from Thermo Fisher Scientific. MTT cell viability assay kit was purchased from Biotium. Deionized water (DI water) was purified using a Millipore Milli-Q system (18 MΩ).

Characterizations. Dynamic light scattering data were measured with a Zetasizer Nano ZS (Malvern, UK). Transmission electron microscopy (TEM) was performed using a Tecnai F30 (FEI Company, USA) equipped with energy dispersive X-ray spectroscopy (EDS, Oxford Instruments) for element analysis and a HT-7700 (Hitachi, Japan) for general imaging purpose. UV-Vis spectra and optical density were taken with an Evolution 220 spectrometer (Thermo Fischer Scientific). Cell cytotoxicity of GSJNPs was analyzed by a plate reader (SpectraMax i3x, Molecular Devices). Ultrasound/photoacoustic (US/PA) images were acquired and analyzed by an integrated ultrasound-photoacoustic imaging system (Vevo LAZR, FUJIFILM VisualSonics, Inc.) equipped with ultrasound transducers (LZ-250 and LZ-550, FUJIFILM VisualSonics, Inc.).

Synthesis of citrate-stabilized GNPs. The synthesis of citrate-stabilized GNPs was achieved as described elsewhere.[1] Briefly, an aqueous citrate solution (2.2 mM, 150 mL) was refluxed by heating in oil-bath. After 10 min, 1 mL of an aqueous solution of $HAuCl_4$ (25 mM) was added to the reaction vessel. The color of the solution turned to blue and then to red. After 20 min of reflux, the temperature of reaction mixture was cooled down until it reached 90 ^oC. To this solution, 1 mL of an aqueous solution of $HAuCl_4$ (25 mM) and 1 mL of aqueous citrate solution (60 mM) were added sequentially (time gap: 2 min) to achieve the seeded growth of GNPs. After 30 min of incubation, the sequential injections were repeated until the size of GNPs reached up to 30 nm in diameter.

Synthesis of GSJNP and GSCSNP. To synthesize GSJNP with 30 nm of GNP, the protocol was modified based on a previous report.[2] The PAA and 4-MBA solutions were prepared in DI water (0.042 mM) and ethanol (5 mM) respectively. A 2-propanol/water co-solvent system was prepared with 38 mL of 2-propanol and 12 mL of DI water. To this solution, 400 μl of both PAA and 4-MBA solutions were added and stirred for 10 min. In parallel, 10 mL of as-prepared citrate-stabilized GNPs were washed with the same amount of DI water. The resulting GNP solution was added dropwise to the reaction mixture of PAA and 4-MBA in 2-propanol/water co-solvent. After 30 min, 1.8 mL of aqueous ammonium hydroxide solution (28-30%) was added to maintain the basic pH, and then 12 mL of TEOS solution in 2-propanol (8.96 mM) was added to form the anisotropic silica nanocoating. The reaction mixture was gently stirred for 12 hours. After the reaction, the products were washed with a 1:1 mixture of 2-propanol and DI water, and filtered using a syringe filter equipped with 0.2 μm pores to remove unwanted aggregates. For GSCSNP, the synthesis was proceeded following the same protocol for GSJNP except the

addition of PAA.

PA imaging with PTFE Tube Phantoms. The aqueous solutions of GSJNP and GSCSNP in DI water were concentrated by centrifugation, and the resulting pellets were re-dispersed in DMEM with 10% FBS (final optical density (OD): 2). Two polytetrafluoroethylene (PTFE) tubes 0.89 mm in diameter (Sub-Lite-Wall®, Zeus Inc.) were filled with 50 μL of GSJNP and GSCSNP solutions, and the resulting tubes were placed horizontally in a water chamber filled with DI water for US/PA imaging characterization. US/PA images of both tubes were acquired simultaneously using an integrated ultrasound-photoacoustic imaging system (Vevo LAZR), with a 20 MHz LZ-250 transducer. Light was delivered via integrated optical fiber, using a nanosecond pulsed laser with 20 Hz repetition rate and an optical parametric oscillator (OPO) emitting from 680 nm to 970 nm. For single wavelength images, 100 frames were acquired at 700 nm excitation and then averaged for signal comparison.

Preparation of GSJNP-Loaded Cells. Two cell lines were chosen to prepare GSJNP-loaded cell samples. Macrophages (J774A.1 cell line) and human breast cancer cells (MDA-MB-231 cell line) were grown in DMEM with 10% FBS to confluency. Upon confluency, a small volume of concentrated GSJNP solution was added into the cell culture medium to fix the final OD of GSJNP at 2.0. The cells were incubated with GSJNP for 48 h at 37 °C and then the GSJNP-loaded cells were collected by centrifugation. The cells were fixed using 10% buffered formalin, and re-dispersed in PBS. The resulting solution of GSJNP-loaded cells was used to create inclusions as part of a gelatin tissue-mimicking phantom.

PA imaging with Tissue-Mimicking Gelatin Inclusions. A tissue-mimicking phantom was

made with aqueous solutions of gelatin and silica gel particles in DI water. The final concentrations of gelatin and silica gel particles were fixed at 6 wt% and 0.2 wt%, respectively, to simulate optical and ultrasound scattering of biological tissues. The solution mixture was heated over 45 °C while mechanical stirring until the solution became homogeneous, and then placed in a vacuum chamber for 5 min for a degassing process. The solution (300 ml) was then poured into a plastic mold and refrigerated overnight to be solidified. The resulting gelatin block was used for the basal structure to support the gelatin inclusions containing GSJNP-loaded cells to be analyzed. The gelatin inclusions were prepared by mixing an aqueous solution containing gelatin (12 wt%) and silica gel particles (0.4 wt%) with an aqueous solution of GSJNP-loaded cells at 1:1 volume ratio. A 15 μL drop of the resulting solution was placed onto the previously made gelatin block to create dome-shaped gelatin inclusions. The resulting phantoms were refrigerated for 4 hours before the US/PA imaging analysis. US/PA images were acquired and analyzed with Vevo LAZR following the same protocol described above, using a 40 MHz LZ-550 transducer.

Characterization of cytotoxicity of GSJNP. Human embryonic kidney cells 293T (HEK 293T) were seeded in a F-bottom 96-well plate at a density of 10,000 cells/well and cultured in DMEM with 10% FBS at 37 °C in 8% CO_2 for 24 h. After aspirating the supernatant of each well, the cells were treated with varying amounts of GSJNP in a total volume of 100 μl of DMEM with 10% FBS for 24 h. 10 μl of MTT solution was added to 100 μl of medium in each well and further incubated at 37 °C in 8% CO_2 for 4 h. 200 μl of DMSO was added to solubilize the mixture and dissolve the formazan salt. The absorbance at 560 nm (signal from blue formazan) was measured with a plate reader and background absorbance at 630 nm was subtracted from the

signal absorbance to obtain normalized absorbance values. All experiments were performed in triplicate. The relative cell viability was normalized relative to the untreated control cells.

PA imaging in vivo. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the Georgia Institute of Technology. Naïve nude mice were anesthetized and injected subcutaneously in the mammary fat pad area with 60 μl of GSJNP or GSCSNP, at an optical density of 4.6 cm^{-1}. Combined ultrasound and multiwavelength photoacoustic imaging was performed at the injection site immediately after, using a Vevo LAZR with 40 MHz LZ-550 transducer. The wavelength acquisition range was from 700 nm to 970 nm, in steps of 2 nm. The particles were allowed to drain for 24 hours to the nearest inguinal lymph node, which was then imaged under anesthesia following the same protocol as day 1. Following imaging experiments the mice were euthanized by CO_2 asphyxiation.

Multiwavelength spectroscopic analysis

Spectroscopic analysis was performed following a previously-reported method[3] based on a linear least squares (LLS) algorithm. *In vivo* PA imaging data were used as input to the unmixing algorithm. The absorption spectra of oxygenated hemoglobin, deoxygenated hemoglobin, together with nanoparticle spectra obtained *in vitro*, were used as parameters.

The output consisted in three image matrices per mouse, corresponding to oxygenated hemoglobin (HbO_2), deoxygenated hemoglobin (Hb), and nanoparticles (GNP). All resulting images were overlaid on the raw ultrasound image using different colormaps.

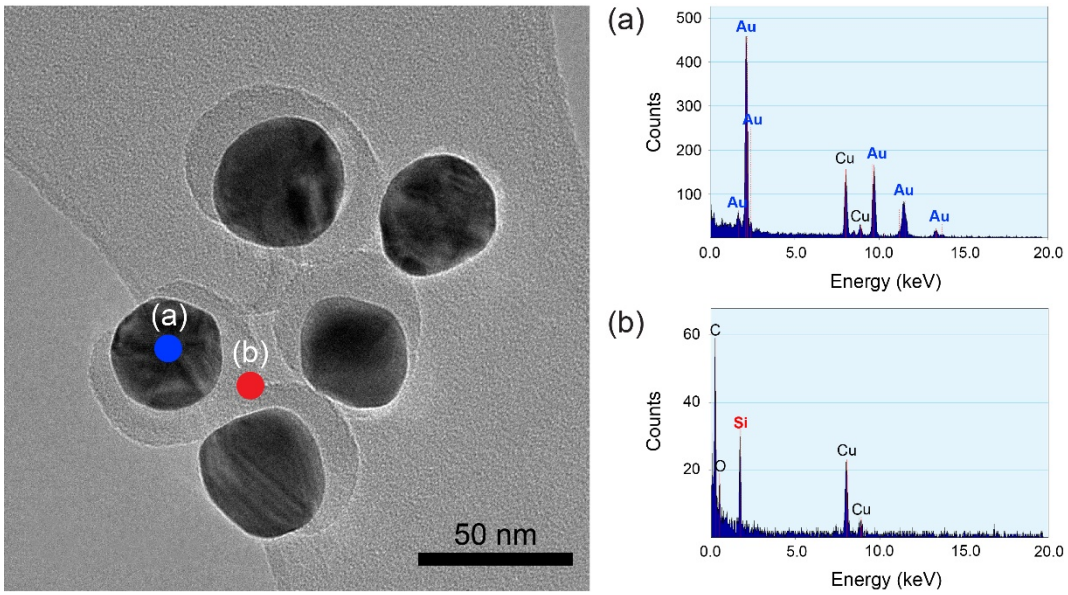

Fig. S1 TEM image of GSJNPs and EDS characterization corresponding to the two spots at (a) gold nanoparticle and (b) silica nanoshell on holey carbon film coated copper grids.

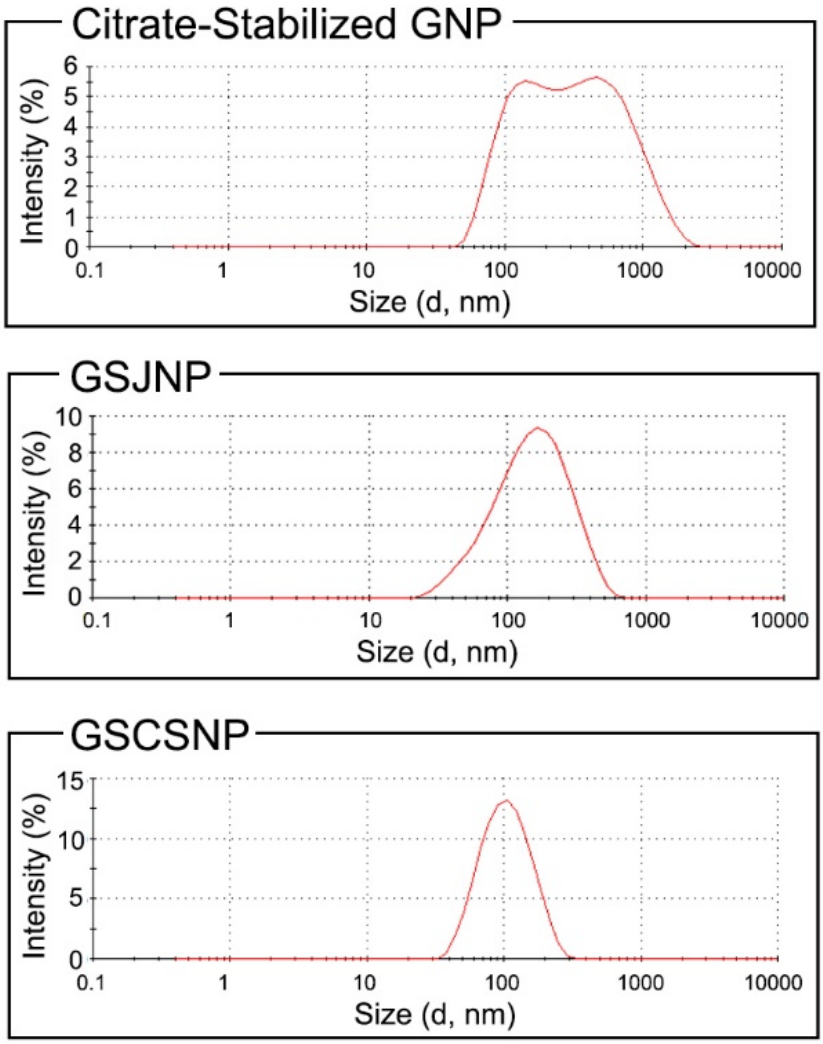

Fig. S2 DLS size distribution curves of citrate-stabilized GNPs, GSJNPs, and GSCSNPs in DMEM with 10% FBS.

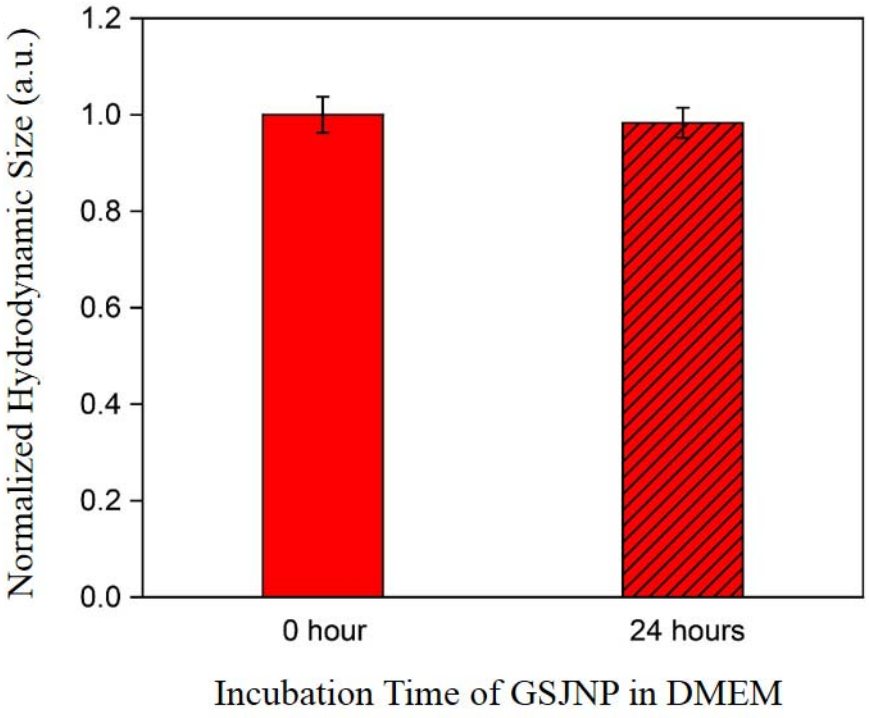

Fig. S3 Stability of GSJNP aggregates in DMEM characterized using DLS.

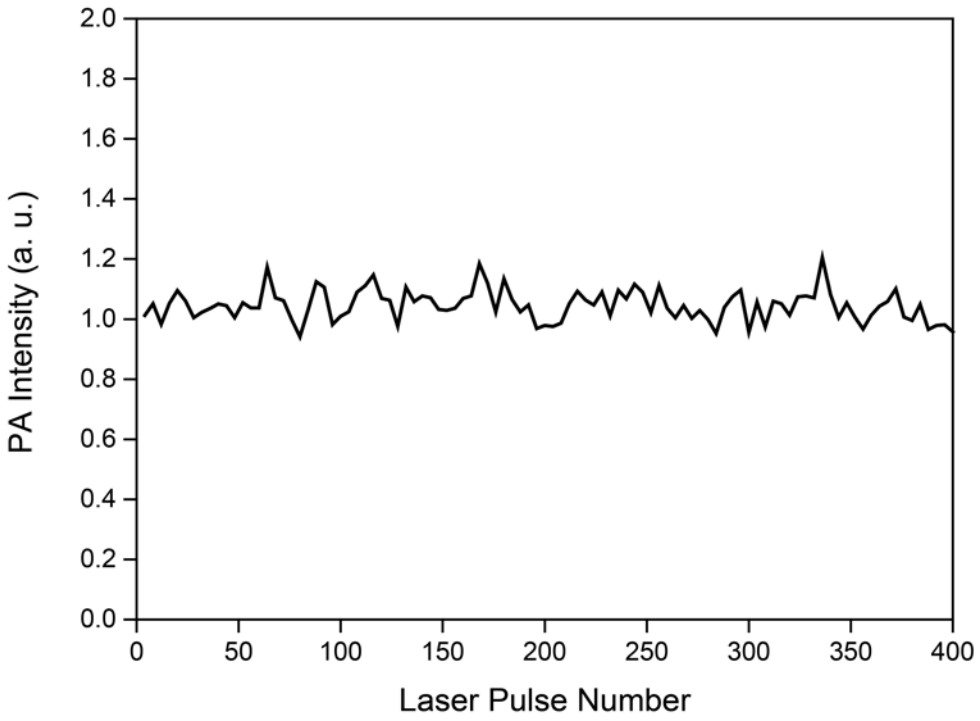

Fig. S4 Stability of PA signal for GSJNPs in macrophages under laser irradiation.

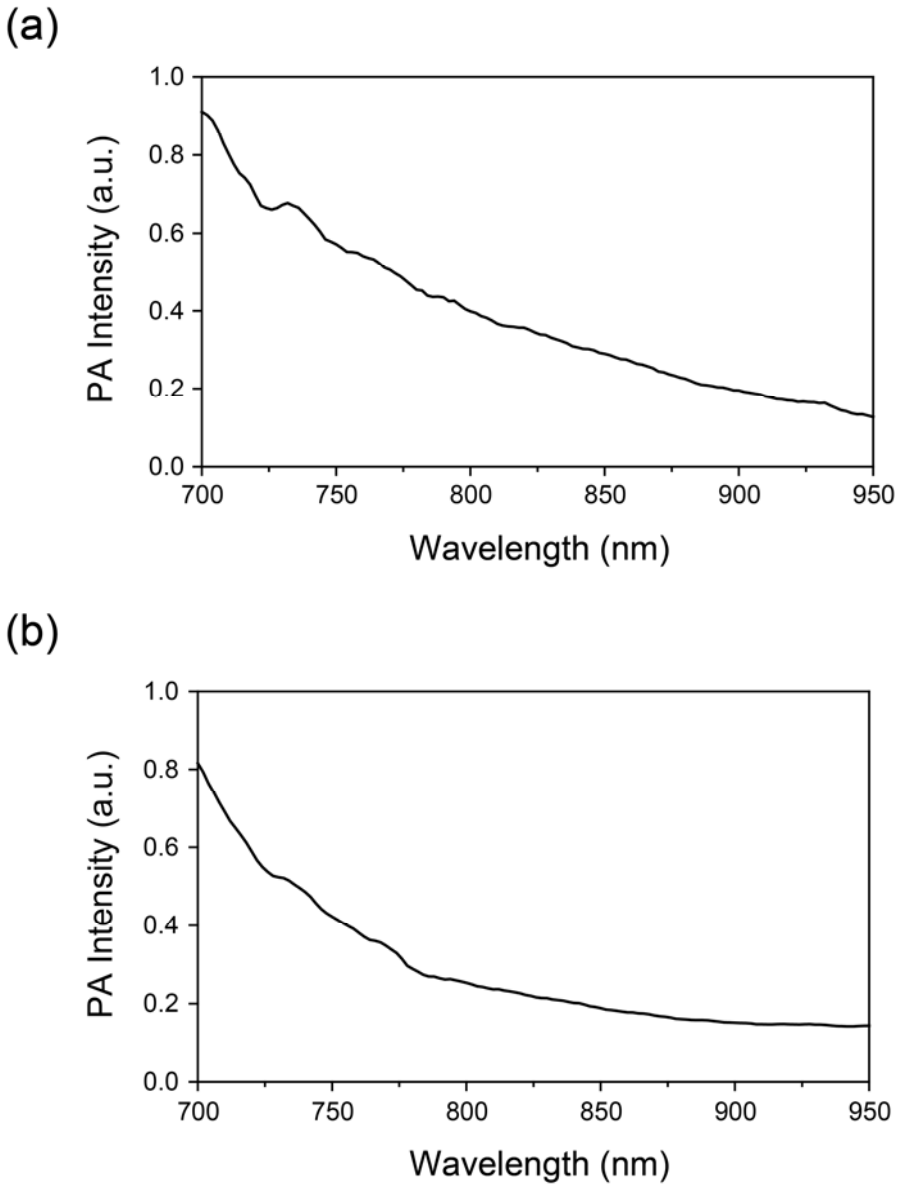

Fig. S5 Multiwavelength PA signal measurements of gelatin inclusions containing (a) macrophages and (b) human breast cancer cells incubated with GSJNPs.

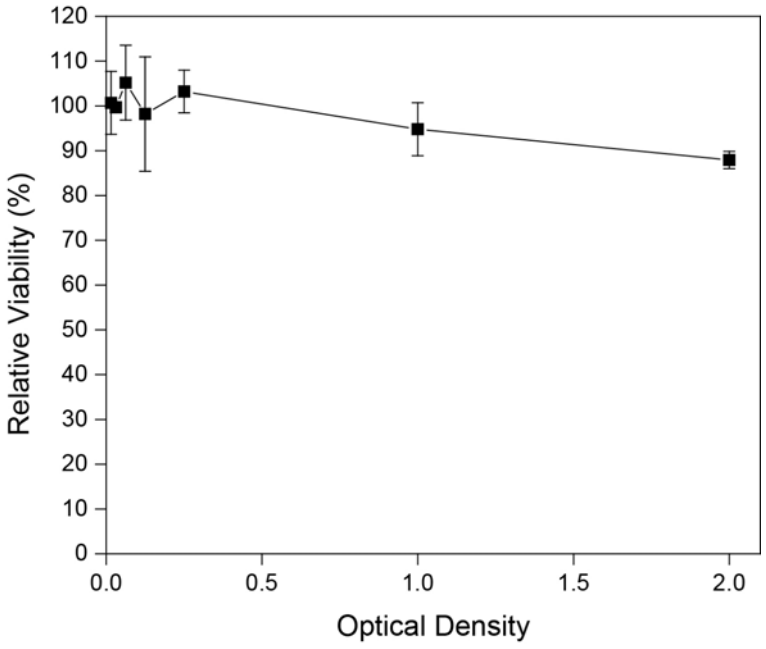

Fig. S6 Cytotoxicity analysis of GSJNPs with HEK 293T cells by MTT assay (y axis: relative % viability calculated by comparing cells treated with GSJNPs with untreated cells, x axis: final OD of GSJNPs at 532 nm).

References

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2. D. Rodríguez-Fernández, T. Altantzis, H. Heidari, S. Bals and L. M. Liz-Marzán, *Chem. Commun*., 2014, **50**, 79–81.
3. S. Kim, Y. S. Chen, G. P. Luke, and S. Y. Emelianov, *Biomed. Opt. Express*, 2011, **2**, 2540-2550.