Standard operating procedures for optoacoustic experiments in phantoms and *in vivo*

Primary sources of variation in optoacoustic image data include hardware instabilities and user operational experience. Hardware instabilities represent the limit of precision of a given optoacoustic system; these are primarily:

1. Light source energy fluctuations, which may arise due to
    a. Lack of temperature stabilization of laser cavity and tuning crystal;
    b. Aging of the pump source;
    c. Dirty or damaged optics;
    d. Occurrence of timing jitters etc.
2. Ambient system noise, which may arise due to
    a. Electronic interference;
    b. Overheating of the control electronics.

User operational experience can impact the precision of a given OT system due to differences in:

1. Coupling efficiency during data acquisition, as the user must ensure that the coupling medium (e.g. water, gel) is free of bubbles and maintained at a similar temperature to the sample.
2. Sample positioning during data acquisition, which can be optimized with the use of motorized translation stages.
3. Region of interest placement during data analysis, which can be minimized by copying regions of interest between data sets.

Intrinsic variations in the sample (e.g. mouse) are frequently the subject of a given study; however, for *in vivo* imaging unwanted variation may also occur as a result of animal handling or physiology, which must be evaluated and accounted for in the given mouse strain, age and gender of interest for an experiment. Data acquisition and

reconstruction parameters are usually treated as variables to be controlled except when they themselves are the subject of the test.

The standard operating procedure presented below has been designed to minimize and quantify the impact of the identified sources of variation.

MATERIALS

Reagents

- Agar (Sigma Aldrich)
- Nigrosin (Sigma Aldrich)
- Intralipid 20% Emulsion (Sigma Aldrich)
- Contrast agents (e.g. Methylene Blue and Cardiogreen, Sigma Aldrich; IR800CW Carboxylate, Licor)
- Glue gun and 3mm polyethylene tubes
- Isoflurane (any vendor)
- Ultrasound gel (Aquasonics, Parker)
- Eye hydration ointment (any vendor)
- Phosphate buffered saline, pH 7.4 (Gibco, Life Technologies)

Equipment

For data acquisition

- Commercial optoacoustic imaging system, including:
    - Tunable pulsed light source;
    - Light delivery optics, which may include fiber bundles, focusing, beam-shaping and beam-sampling optics;
    - Ultrasound transducer array;
    - Motorized translation stages and suitable controllers, if required;

    - Spectrometer and power meter for routine monitoring of (and offline correction for) laser energy output;
    - Data acquisition system with signal conditioning units;
    - Reconstruction and data analysis computer;
    - Instrument control, reconstruction and analysis software.
- Phantom and animal holders to provide repeatable positioning of the sample.

For phantom preparation

- Weighing scales or balance.
- Microwave oven.
- Warm water bath operating at up to 60°C.
- Spectrophotometer with the same wavelength range as the tunable pulsed light source.
- Phantom molds (shape determined by the optoacoustic imaging system geometry).
    - Molds can be easily formed in desired geometry using a 3D printer.
- Polyethylene tubes (with minimum wall thickness and of a suitable diameter for the given optoacoustic imaging system geometry).

For animal experiments

- Inhalation anesthesia delivery system.
- Heating system.
- Venous catheter for intravenous contrast agent administration.
- Polyethylene membrane, to avoid direct contact with animal during imaging procedure.
- Ideally, a physiology monitoring system would be used to assess temperature, respiration and pulse oximetry.

Samples

- Commercial stable polyurethane phantom (fabricated by Computerized Imaging Reference Systems Inc., supplied by iThera Medical)
- Agarose phantoms (see below)
- Healthy BALB/c nude mice (~ 18 g, Charles River). Full details of the housing conditions for the mice are given in the main article.
    - Although immune compromised mice were used for our studies, other strains of mice could be used upon hair removal around the region of interest.
    - Replicates should be performed with the same strain, age and health condition.

PROCEDURES

Tissue mimicking phantom fabrication

Recipe to make 100 mL solution with 0.05 cm^{-1} absorption coefficient and 5 cm^{-1} scattering coefficient

- Weigh 15 mg of nigrosin and add to 30 mL deionised water in a 50 mL falcon tube. Measure the absorption spectrum of the solution using a spectrophotometer and ensure that a 1:2 dilution series in additional deionised water maintains linearity.
- Measure 2.08 mL of intralipid into a 15 mL falcon tube and pre-warm in a water bath at 50°C for at least 5 minutes.
- Weigh 1.5 g of agar and add to 97.3 mL deionised water in a glass media bottle. With the cap placed loosely on the bottle, heat the solution inside a microwave

oven for around 1 minute (depending on power rating), until it visibly boils and the solution appears clear

- *Important, do not let the solution overflow the flask.*

- Allow the solution to cool down to less than 60°C and add the pre-warmed intralipid into the agar solution.
- Immediately measure 0.62 mL of the nigrosin solution and mix thoroughly to form a homogenous solution.
- Immediately pour the solution into suitable phantom molds and leave the solution to set in room temperature or inside a refrigerator
    - *Important, always use the same method for allowing the phantom to set; room temperature or refrigeration may result in different phantom properties.*

Preparing for optoacoustic data acquisition

The optoacoustic imaging system (including light source and tuning units, as well as any coupling water bath) should be switched on sufficiently far in advance of the imaging session to allow adequate warm-up time of the given light source, according to the suggestion of the manufacturers. Typical values range from 15-30 minutes. All acquisition hardware and any motorized translation stages should be initialized and checked prior to data acquisition.

Data acquisition variables should be identified for the optoacoustic imaging system under test. In this study, we examined the effects of changing wavelength, rotational sampling positioning and signal averaging. Other variables that could be tested, but were not relevant for our system, include: number of views; ultrasound frequency (if multiple handheld probes are available, for instance); and gain / time gain compensation (particularly in linear transducer arrays). Each variable should be adjusted

in turn and image data recorded from a stable phantom. Once these data have been evaluated, a defined imaging protocol should be set for an entire series of comparable samples (e.g. a longitudinal phantom or in vivo study).

Phantom imaging

Phantoms may be composed of a stable material (such as the commercial polyurethane phantom used in this study, or a photostable material such as a carbon fiber) or a tissue mimicking material (as found in the above recipe). Images should be periodically acquired from a stable phantom to verify that the OT system is meeting performance expectations. Tissue mimicking phantoms are used to evaluate the system sensitivity and precision for detection of contrast agents, to assess the dose that should be used for *in vivo* experiments. In the latter case, a dilution series of the chosen contrast agent (such as the small molecule dyes detailed in the materials section above) should be prepared in appropriate solvent and encapsulated into polyethylene tubes, using a glue gun to seal the tubes. The dye-filled tubes can be exchanged as required between the same phantom. Phantom imaging then proceeds as follows:

1. Place the phantom within the field of view of the OT system;
    a. Placement is best achieved using a bespoke phantom holder that will allow repeatable sample positioning between measurements.
2. Allow the phantom temperature to stabilize with the surroundings, in our experience for at least 15 minutes (longer if removing from the refrigerator). If required, apply bubble free ultrasound gel or water to couple the phantom to the ultrasound transducers.
3. Identify the regions of interest using an appropriate prescan or ‘live view’ mode. Fix the position, or if required, set translation stages to scan across multiple positions.

4. Start data acquisition.

In vivo imaging

The impact of anesthesia on the recorded signals from oxy- and deoxy-hemoglobin is significant in the organs that we tested in this study.

1. Bring animal cage to preparation area for the optoacoustic imaging system.
2. Induce anesthesia in a warm induction chamber using inhaled isoflurane at up to 3% mixed with 100% oxygen gas. Reduce isoflurane to <2% for maintenance.
3. Apply eye hydration ointment to avoid dry eyes and if in the field of view of the laser, gently apply surgical tape over the eyes.
4. For studies involving contrast agents, prepare venous catheters with identical lengths for all replicates and retain the position of the catheter inside the tail vein using suitable tissue glue.
5. Apply chosen coupling medium to the mouse (bubble free water or a thin layer of ultrasound gel) to enable good acoustic coupling.
6. Place the mouse into the OT system.
    a. *Important, use a fixed preparation time from induction of anesthesia to placement into the system. In our case this was ~ 15 min.*
7. Allow the mouse body temperature (assessed by rectal probe) to stabilize in the physiologically normal range (36-38°C).
    a. *Important, temperature can impact respiratory rate and hence influence recorded oxy- and deoxy-hemoglobin values.*
    b. Adjust the isoflurane concentration during the recovery period (~12 min in our OT system) to maintain a respiration rate of 60-70 bpm (~1.75±0.25% isoflurane in this study).
    c. During this equilibration period, additional setup tasks can be performed:

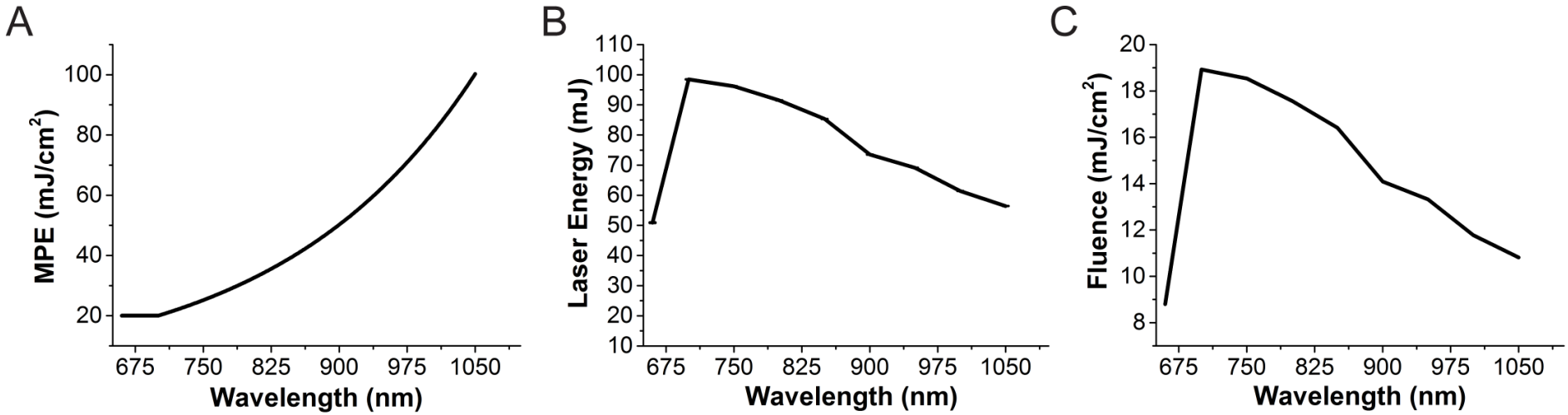
i. Check for artifacts (e.g. streaks) due to air bubbles and if found, repeat coupling and placement procedure.
ii. Identify the regions of interest to scan and if required, set translation stages to scan across multiple positions.
iii. For longitudinal imaging of a given organ, noting the position of the organ extrema and setting a central slice position can be helpful to achieve consistent anatomical positions between each replacement.

8. Start the data acquisition.
9. For reproducibility studies relating to contrast agent administration, maintain identical volumes of administration and similar perfusion time for each injection.
10. Remove the mouse at study completion, or before the imaging time duration approved by local and national animal welfare bodies is exceeded, and allow to recover in a heated recovery box.

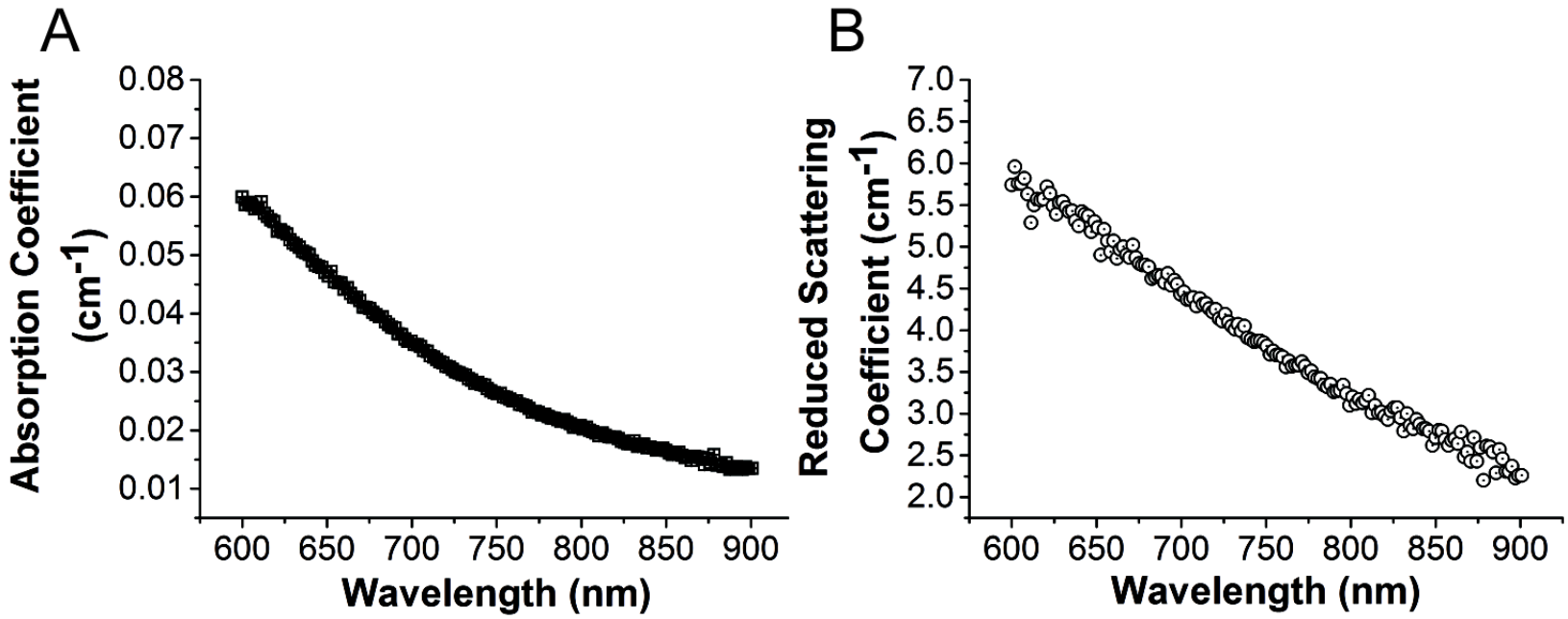
Offline image reconstruction and data analysis

Optoacoustic signals should first be corrected for the measured illumination energy to account for any wavelength-dependent variation in excitation energy. Where available, spatial and/or electrical impulse response functions for the ultrasound transducers may also be applied prior to reconstruction to account for imperfections in the ultrasound detection. All image reconstruction and analysis variables should be identified and their impact systematically evaluated for the OT system under test using an image of a stable phantom containing variable feature sizes. Where relevant, the reconstruction procedure for a given study should be applied with a consistent set of parameters, which may include: algorithm choice (e.g. backprojection or model-based); digital filters (and associated frequency settings); speed of sound in the sample; and scan conversion

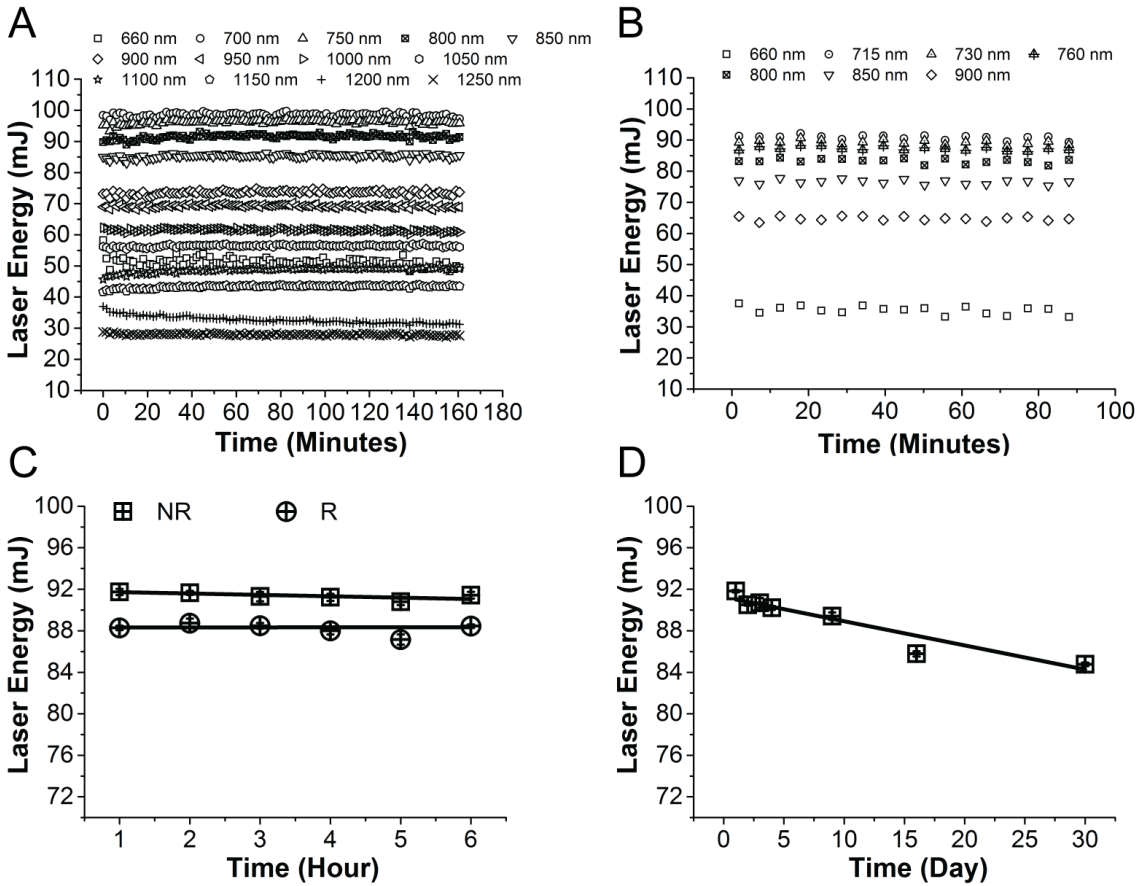
settings. The speed of sound has a significant impact on the focus of the image, so if the value for a given sample is unknown, it may need to be determined empirically in the reconstruction process. To extract quantitative data, consistent ROIs with a predefined size and location should be applied to the image and ROI statistics should be exported.



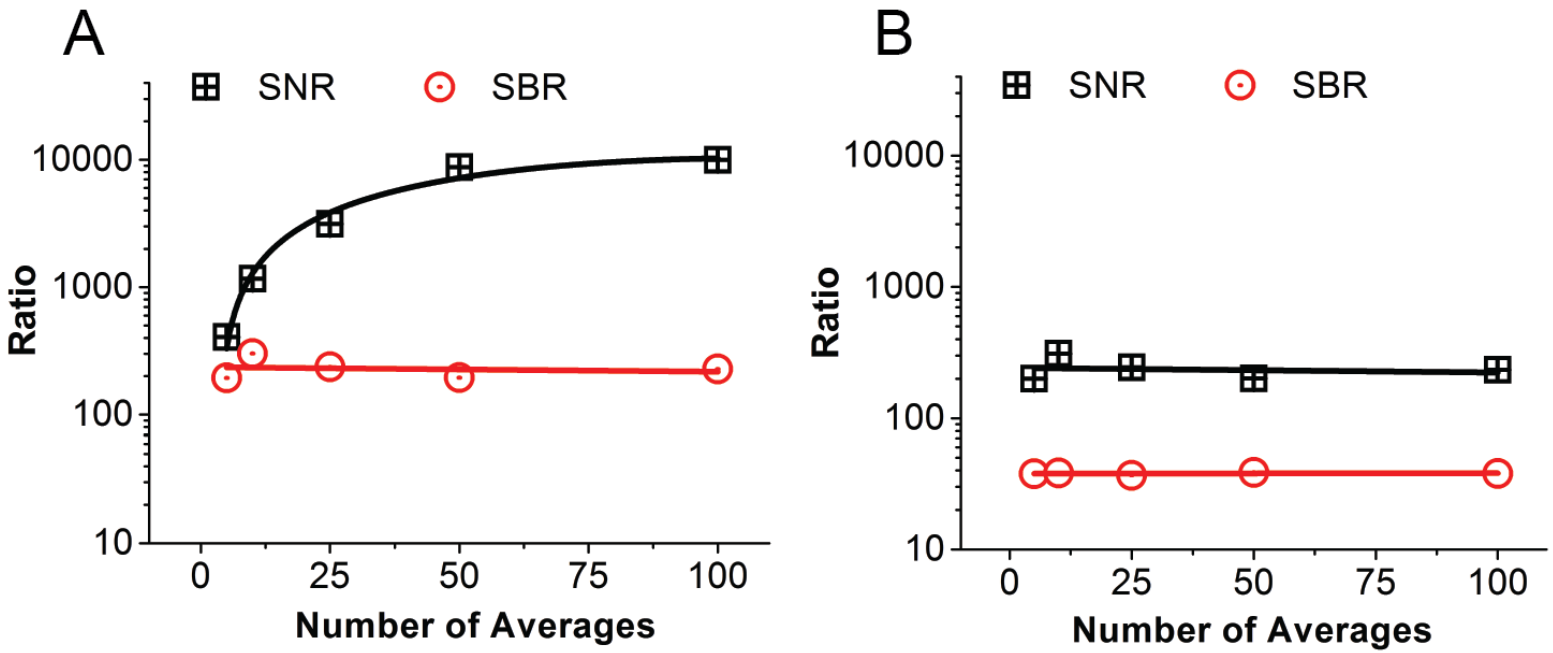
Supplemental Figure 1. Maximal permissible exposure (MPE) for skin and laser energy output over the wavelength range of interest. (A) MPE calculated according to BS EN 60825-1:1994 with IEC 60825-1 amendment 2 2001-01. (B) Laser energy measured before coupling light into the delivery fibers. (C) Corresponding light fluence at the sample, assuming the irradiated area to be the surface of a cylinder with height 8 mm (measured width of illumination) and radius 4 mm, given a beam diameter at the output of the OPO of 8.2 mm.



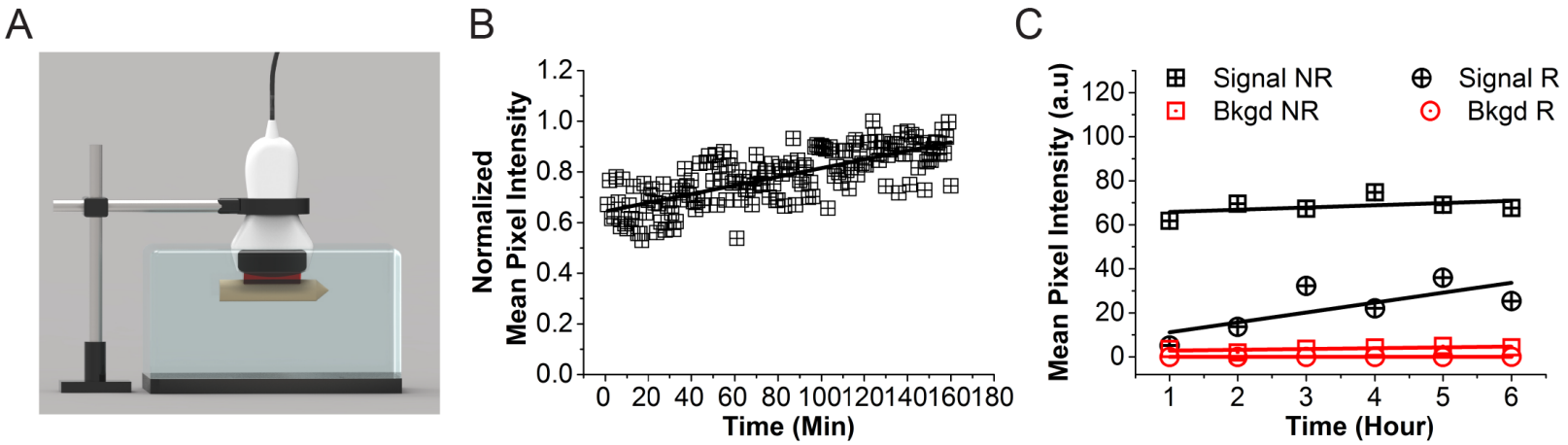
Supplemental Figure 2. Measured absorption and reduced scattering coefficients for the agarose gel composing tissue mimicking phantoms. The absorption (A) and reduced scattering (B) coefficients in the phantoms were selected based on the generic tissue optical absorption, and scattering coefficients were provided for the purpose of testing devices or for protocol design by Jacques (1). The lowest suggested reduced scattering coefficient from Jacques (1), Mie 5 cm^{-1}, was chosen to facilitate direct comparison between the limits of detection measured in phantoms and *in vivo*. Data were acquired using a double integrating sphere system, implemented according to published methods and calibrated against spectrophotometer measurements (2, 3). Error bars are within the points.



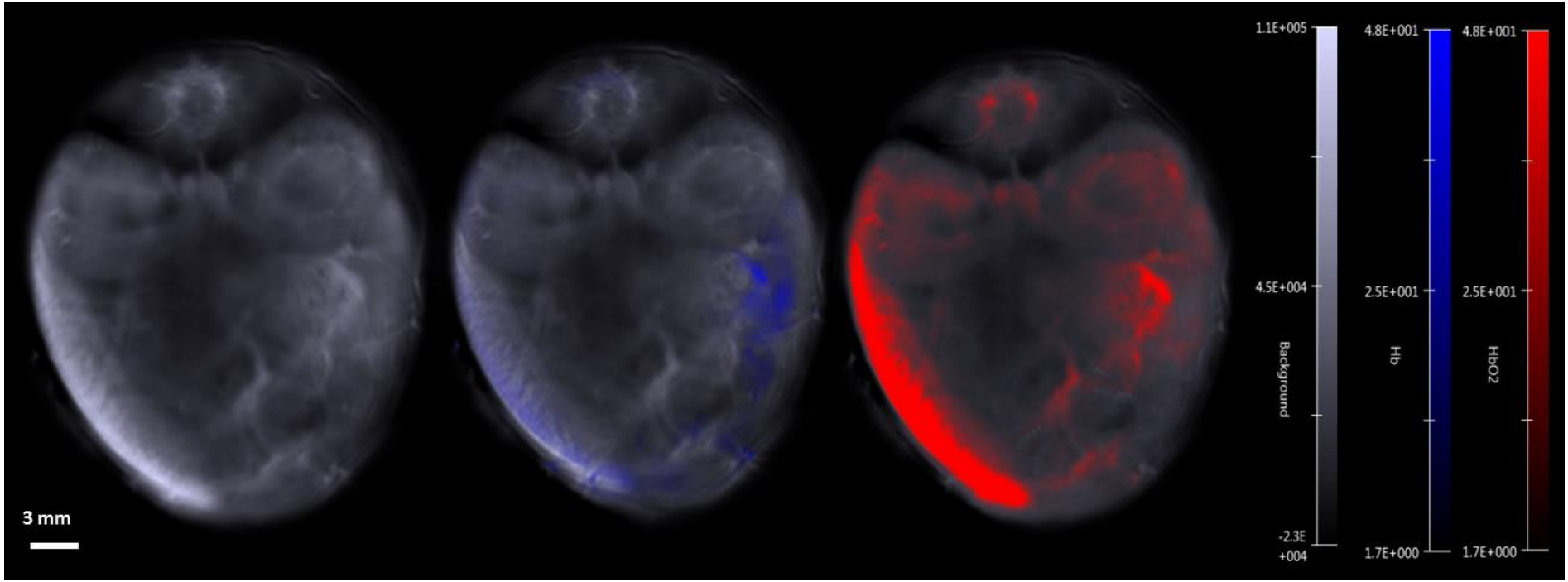
Supplemental Figure 3. Laser energy for repeatability studies. Laser energy (measured before coupling light into the delivery fibers per Figure S1B) for each wavelength used in the phantom studies shown in Figure 2 of the main article over 160 minutes (A) and in the small animal repeatability study shown in Figure 4(A) of the main article over 90 minutes (B). Laser energy at 700 nm for (C) 6 hour stability studies (Figure 2B) and (D) 30 day stability studies (Figure 2C); linear fits give slopes of (-13.35±6.37)E-2 mJ hour^{-1} for no replacement, (0.43±7.29)E-2 mJ hour^{-1} for replacement for (C) and (-23.27±3.15)E-2 mJ day^{-1} for (D).



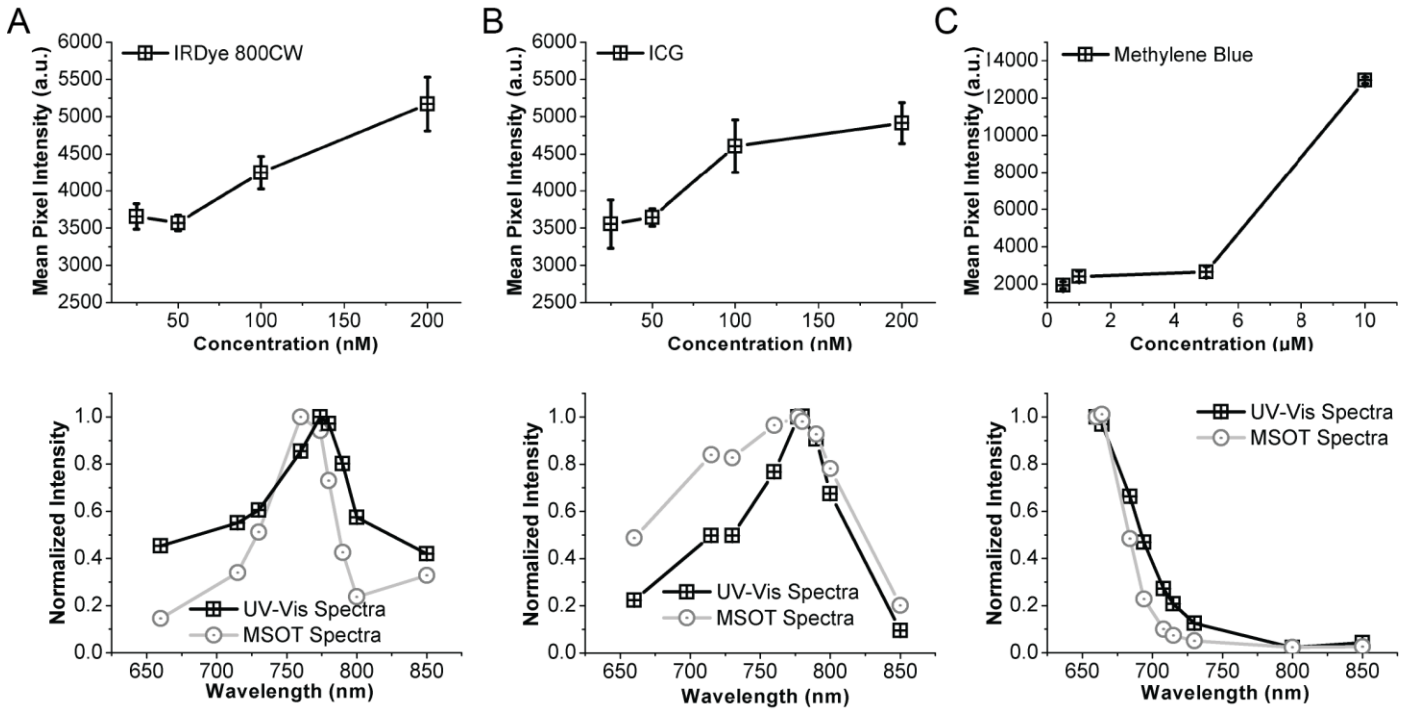
Supplemental Figure 4. Impact of frame averaging on image quality. Imaging signal-to-noise ratio (SNR) and signal-to-background ratio (SBR) as a function of the number of frame averages performed in sequential mode (A) or continuous mode (B). We calculated both parameters since we expect SNR to increase with averaging while SBR should remain constant, hence providing us with a reference. Continuous acoustic signal averaging does not influence image SNR or SBR. Polynomial fit of SNR in (A) gives B1 value of (44.35E-3±9.18E-18) and B2 value of (-2.97E-4±8.51E-20) and linear fit for SBR in (A) gives a slope of (-3.6E-4±11.4E-4) for sequential mode. Linear fits of continuous mode averaging in (B) give slopes of (-3.61E-4±1.15E-3) for SNR and (4.4E-5±1.24E-4) for SBR. Error bars are within the symbols.



Supplemental Figure 5. Optoacoustic precision as a function of time in the stable polyurethane phantom at 850 nm measured with an LED-based optoacoustic imaging system. (A) Schematic of the optoacoustic imaging system, reported in (4), and the experimental setup for phantom measurements. Briefly, optoacoustic signals are excited by a 70 ns pulsed light emitting diode array at 850 nm with a 4 kHz repetition rate and detected by a 128-element linear array transducer (9 MHz center frequency). For each excitation pulse, optoacoustic images were reconstructed with 320 signal averages. The standard operating procedure was applied to the same commercial polyurethane phantom used in the rest of this study, using a water bath for ultrasound coupling. (B) Normalized mean pixel intensity over 160 mins exhibits a slope of (0.64±0.01) a.u. and COV of 13.9%. The upward drift may occur due to heating of the light emitting diode arrays and water medium, as the system does not currently apply per pulse energy compensation. (C) Mean pixel intensity (arbitrary units, a.u.) over 6 h in a single day with replacement (R) and without replacement (NR) of the phantom between data acquisitions; slopes are (6.67±8.18) a.u. and (64.81±3.88) a.u. respectively. The COV was 6.1% over 6 h (NR) rising to 51.3% (R) when the phantom was removed between data acquisitions, due to the difficulty of achieving repeatable phantom positioning.



Supplemental Figure 6. Example images of Hb (blue) and HbO_2 (red) signals. Color scales are in arbitrary units and represent the hemoglobin weighted signals derived from spectral unmixing (see Section 2.3).



Supplemental Figure 7. Sensitivity and spectral response for three commonly used dyes in tissue mimicking phantoms. Detection limits at ~1 cm depth in a tissue mimicking phantom plotted as the mean pixel intensity at the peak absorption wavelength, along with absorption spectra of the NIR dyes IRDye 800 CW (A); ICG (B); and Methylene Blue (C). IRDye 800CW (IR800, Licor) and indocyanine green (ICG) were tested in concentrations ranging from 1 nM to 1 µM. Methylene blue (MB) was tested in the range from 1 nM to 50 µM. Concentration ranges relevant to the detection limit are plotted for clarity. The limit of detection was assessed by comparing the data from n=5 replicates at each dilution (from solvent only at 0 nM then 1 nM upwards) using a t-test until the p value for comparison of two successive dilutions was less than 0.05. The excitation wavelengths used were chosen to accurately sample the expected excitation spectrum of the dyes according to the manufacturer published profiles: IR800: 660, 715, 730, 760, 774, 780, 790, 800 and 850 nm; ICG: 660, 715, 730, 760, 777, 780, 790, 800 and 850 nm; MB: 660, 664, 684, 694, 708, 715, 730, 800 and 850 nm. Dye characteristics were verified independently using a UV-Vis plate reader (PHERAstar FS, BMG Labtek GmbH) and the measured spectra are shown for reference. Data represent an average of n=5 MSOT scan positions per time point and error bars are within the symbols.

Supplemental Table 1: Coefficient of variation of MSOT signal intensity for the polyurethane stable phantom. Data is shown as function of excitation wavelength over 1 day, 1 week and 1 month and also for different rotational positions (see Figure 3). Larger coefficients of variation at longer wavelengths are due to increased light absorption in the water bath used for acoustic coupling.

	Coefficient of Variation (%)									
λ (nm):	660	700	750	800	850	900	950	1000	1050	1100
1 day (n=6) (with replacement)	1.2	1.2	1.3	1.3	1.4	1.3	1.8	2.8	1.7	0.9
1 day (n=6) (no replacement)	0.6	0.5	0.7	0.5	0.6	1.0	0.8	1.0	1.1	0.7
1 week (n=4)	2.0	1.9	2.0	1.9	2.0	1.9	1.7	1.6	1.6	2.2
1 month (n=7)	1.9	1.9	2.1	1.9	2.0	2.4	1.6	1.6	2.2	2.1
Positioning (n=4)	3.6	3.6	4.0	4.3	4.3	4.0	8.1	10.1	4.0	4.6

References

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