**Grueneisen relaxation photoacoustic microscopy: supplementary materials**

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**Validation of the Grueneisen relaxation effect**

The Grueneisen relaxation effect was experimentally validated by imaging a red blood cell (RBC) sample. A monolayer of RBCs was fixed on a glass slide, immersed into deionized water, and positioned in the optical focal plane. We acquired multiple PA images using two-dimensional raster scanning. At each measurement, the PA amplitude was calculated from the average signal of the entire PA image.

First, we validated the linear relationship between the single-pulse PA amplitude and the excitation laser pulse energy. To avoid the Grueneisen relaxation effect, only one laser was triggered at 10 kHz. Laser energy was set between 0 to 60 nJ. Supplementary figure 1a shows that the single-pulse PA amplitude was proportional to the laser pulse energy.



Supplementary figure 1. (a) Linear relationship between single-pulse PA amplitude and pulse energy. (b) Linear relationship between the increase in the second PA amplitude and the first laser pulse energy. The first laser pulse energy varied from 0 to 25 nJ. The second laser pulse energy was set at 25 nJ. The two laser pulses were separated by 500 ns delay. ΔPA2 = PA2 – PA2\_0. PA2 was the second PA amplitude, whereas PA2\_0 was the baseline of the second PA amplitude measured when the first laser pulse energy was zero. (c) The increase of the second PA amplitude versus inter-pulse time delay while both laser pulse energies were 25 nJ.

Then the Grueneisen relaxation effect was experimentally demonstrated using two lasers that were sequentially triggered with a 500 ns delay. Both laser pulse energies were chosen within the validated linear PA range. The first laser pulse energy was varied from 0 to 25 nJ, while the second laser pulse energy was held at 25 nJ. The second laser PA amplitude (PA2) increased from its baseline (PA2\_0) as we increased the first laser pulse energy from 0 to 25 nJ. Supplementary figure 1b shows a linear relationship between the increase of the second laser PA amplitude and the first laser pulse energy. The largest increase of the second PA amplitude was 14%. According to the linear relationship between water’s Grueneisen parameter and temperature [1], the highest temperature rise was estimated to be ~3 oC .

We also tested the dependence of the Grueneisen relaxation effect on the time delay between the two pulses. Both of the laser pulse energies were set to 25 nJ. The time delay was tuned from 75 ns to 5 µs. As shown in Supplementary figure 1c, the amplitude increase of the second PA signal follows an exponential function of the time delay due to thermal relaxation.

Reference

[1] L. Gao *et al.*, Journal of Biomedical Optics **18**, 026003 (2013).