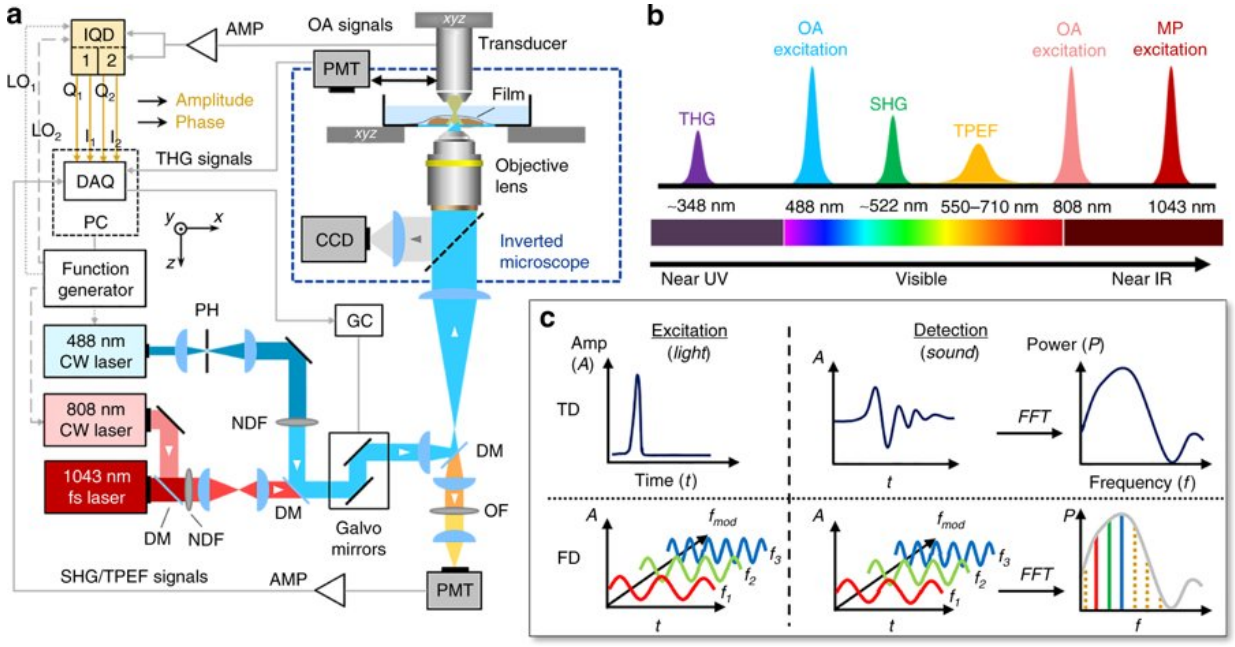
Optoacoustic microscopy at multiple discrete frequencies

January 9 2019, by Thamarasee Jeewandara



Schematic representation of the hybrid microscopy system containing a subsystem for dual wavelength optoacoustic microscopy at 488 nm and 808 nm, co-aligned with a subsystem for multiphoton microscopy at 1043 nm. a) AMP amplifier, CCD bright-field camera, DAQ data acquisition card, DM dichroic mirror, GC galvanometric mirror controller, IQD IQ demodulator, LO1 local oscillator 1, LO2 local oscillator 2, NDF neutral density filters, OA optoacoustic, OF optical filter, PC personal computer, PH pinhole, PMT photomultiplier tube, SHG second-harmonic generation, THG third-harmonic generation, TPEF two-photon excitation fluorescence, xyz motorized stages. b) The spectrum of the excitation and detection wavelengths in hybrid FDOM/multiphoton (MP) imaging. c) Schematic comparison between time-domain (TD) optoacoustic microscopy, which uses short pulses of light, and frequency-domain (FD)

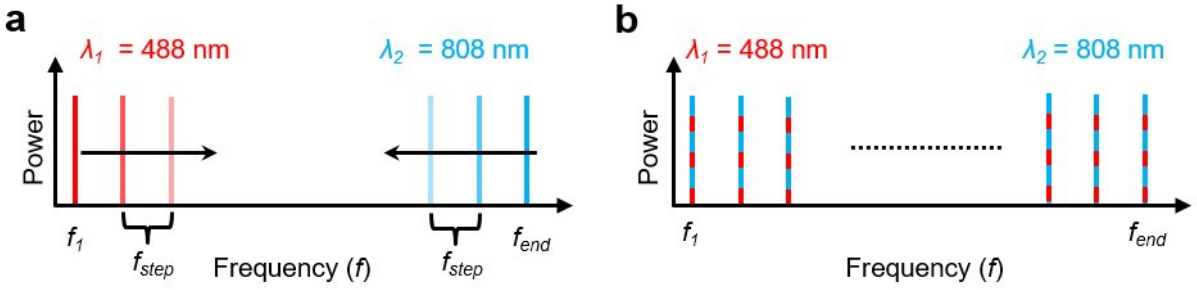
optoacoustic microscopy, which is based on laser intensity modulated at multiple discrete frequencies. Credit: *Light: Science & Applications*. Doi: https://doi.org/10.1038/s41377-018-0101-2

Optoacoustic imaging powered by short bursts of continuous wave (CW) lasers can stimulate the emission of ultrasound waves inside an animal or in human subjects. The method can noninvasively capture blood flow and produce 3-D images of cellular microarchitecture. Writing in *Light: Science & Applications*, Stephan Kellnberger and colleagues at the Institute of Biological and Medical Imaging, now report the possibility of obtaining high-fidelity optoacoustic images with cost-effective lasers controlled at multiple frequencies.

The authors experimentally demonstrated the multiple frequency-based, high-fidelity image generation of biological architecture by imaging fish and mouse tissue microvasculature. In the imaging experiments, they superimposed structural details that only appeared at specific frequencies of interest. The authors also non-invasively identified the speed of blood flow in tissue microvasculature by tracking the frequency shifts using the optoacoustic Doppler Effect.

Optoacoustic (photoacoustic) sensing usually requires complex laser technologies. Such techniques can generate nanosecond length (1-100 ns), high-energy short photon pulses that conventionally illuminate transient (short-lived) energy in the time domain (TD). The ultra-short pulses can stimulate the emission of broadband ultrasonic waves, collected in the microsecond range to form optoacoustic images. However, complex laser technology can impose a low-pulse repetition frequency (PRF) and limit the number of wavelengths simultaneously available for spectral imaging. To avoid such limits, Kellnberger et al. developed frequency-domain optoacoustic microscopy (FDOM), in

which light intensity can be controlled or modulated at multiple discrete frequencies using cost-effective hardware.



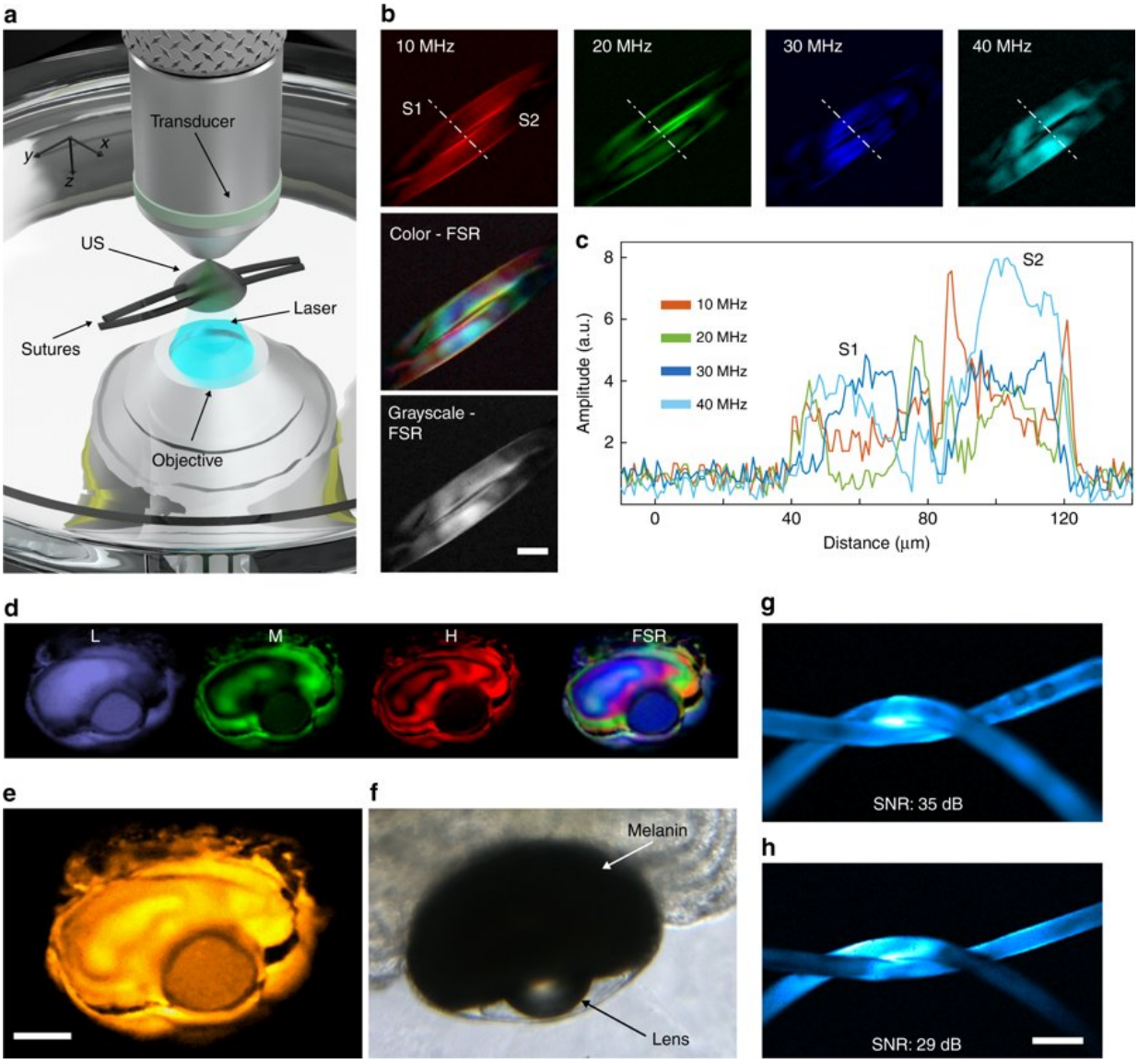
Explanation of frequency coding in dual wavelength FDOM. a) Simplified schematic of frequency coding on different wavelengths. Laser source 1 emitting at λ1 = 488 nm was loaded with the lowest modulation frequency f1, while laser source 2 emitting at λ2 = 808 nm was loaded with the highest modulation frequency fend. During imaging, we increased the modulation of wavelength λ1 and decreased the modulation frequency of λ2 in steps of fstep using odd numbers of modulation frequencies. b) Schematic representation of multiple modulation frequencies used for imaging, showing the superposition of frequencies at two wavelengths. Credit: *Light: Science & Applications*. Doi: https://doi.org/10.1038/s41377-018-0101-2

Thus far, optoacoustic imaging has only relied on techniques that detect signals in the time domain (TD) or those that only scan a single frequency at one or two wavelengths in the frequency domain (FD). The present study was a first to conduct in vivo optoacoustic imaging in an animal model via simultaneous illumination with two wavelengths.

The scientists combined FDOM into a hybrid system to examine the relationship between image formation and frequency control. The use of discrete frequencies (a maximum of nine), allowed non-invasive

optoacoustic Doppler shift measurements as flow observations in a microfluidic flow chamber in the lab first, and in tissue microvasculature in vivo thereafter. In the study, Kellnberger et al. used two CW diode lasers emitting light at 488 nm and 808 nm for illumination.

The scientists implemented the FDOM, operating in the frequency range of 5-50 MHz, as a hybrid system with multiphoton (MP) microscopy operating at 1043 nm. They then performed two-/three-dimensional imaging based on ultrasound amplitude and phase measurements at multiple frequencies. The amplitude and phase of the generated optoacoustic signals were resolved via demodulation in real time and recorded using an analog-to-digital converter. Due to high repetition rates, the FDOM achieved high signal-to-noise ratios (SNR), leading to the observed high-fidelity images. In total, the study examined the relationship between the modulation frequency, image fidelity and the signal-to-noise-ratio (SNR).



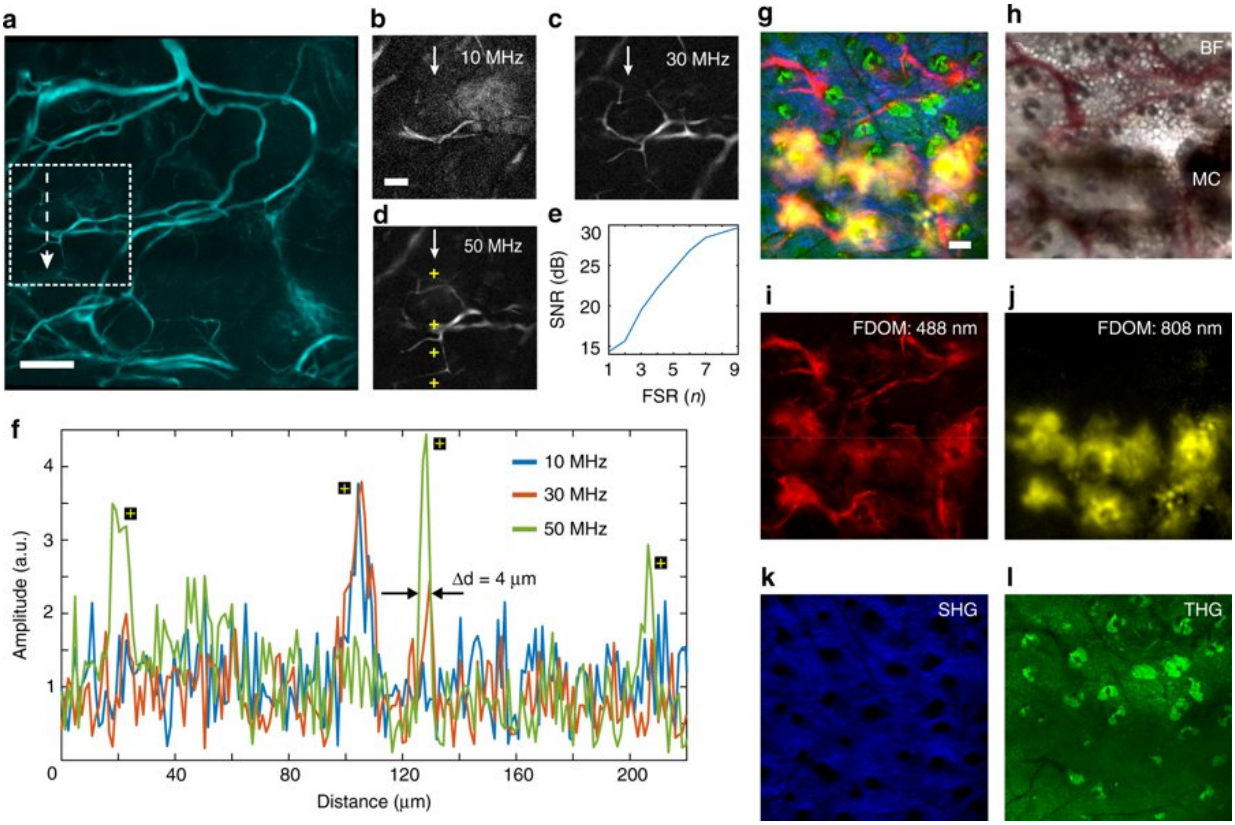
Single-wavelength FDOM imaging of a suture and ex vivo Zebrafish samples. a) A schematic illustration of the scanning of two crossing sutures. b) Color-coded FDOM images of two 50-μm sutures, based on illumination at 488 nm and modulation frequencies of 10, 20, 30, and 40 MHz. The color frequency-space representation (FSR) superimposes the contributions by each modulation frequency. The grayscale FSR image based on four frequencies shows the final image. c) Cross-sectional profile of the dashed line shown in panel b, which compares the contrasts revealed by the various modulation frequencies. d) Ex vivo imaging of a zebrafish larva eyeball. The purple image was reconstructed using low (L) frequencies (10, 15, and 20 MHz); the green image using middle

(M) frequencies (25, 30, and 35 MHz); and the red image using high (H) frequencies (40, 45, and 50 MHz). The color-coded overlay of all frequencies (FSR, 10 to 50 MHz) highlights the contribution of each spectral region. e) Orange color depicts the amplitude sum for the nine employed modulation frequencies. f) A bright-field image of a zebrafish eye, validating the fidelity of FDOM images. g) A comparison of the signal-to-noise ratios (SNRs) of images of two crossing sutures (40 μm diameter) obtained via FD and TD optoacoustic microscopy. The FDOM image yielded an SNR of ~35 dB. h) Under similar experimental settings, TD microscopy resulted in an SNR of ~29 dB. Credit: *Light: Science & Applications*. Doi: https://doi.org/10.1038/s41377-018-0101-2

To identify the characteristics of FD photoacoustic imaging, the scientists imaged a pair of crossed sutures in water at two wavelengths (488 nm and 808 nm) and discrete modulation frequencies. The superposition of various frequency contributions carried information of the imaged object (sutures).

To extract information from more complex structures, Kellnberger et al. imaged the eye of 5-day-old wild-type Zebrafish lava ex vivo, using nine modulation frequencies spanning 10-50 MHz in 5-MHz steps. The scientists also compared the SNR (signal-to-noise ratio) between the FDOM method and conventional TD, which varied according to experimental parameters (laser energy, power employed and data acquisition hardware).

Multifrequency amplitude and phase data could thus be processed for 3-D image reconstruction using a Fourier transform based on frequency-space representation (FSR) and time-space representation (TSR). Compared with TSR, the FSR based image reconstruction was computationally faster and did not require data inversion during image reconstruction.



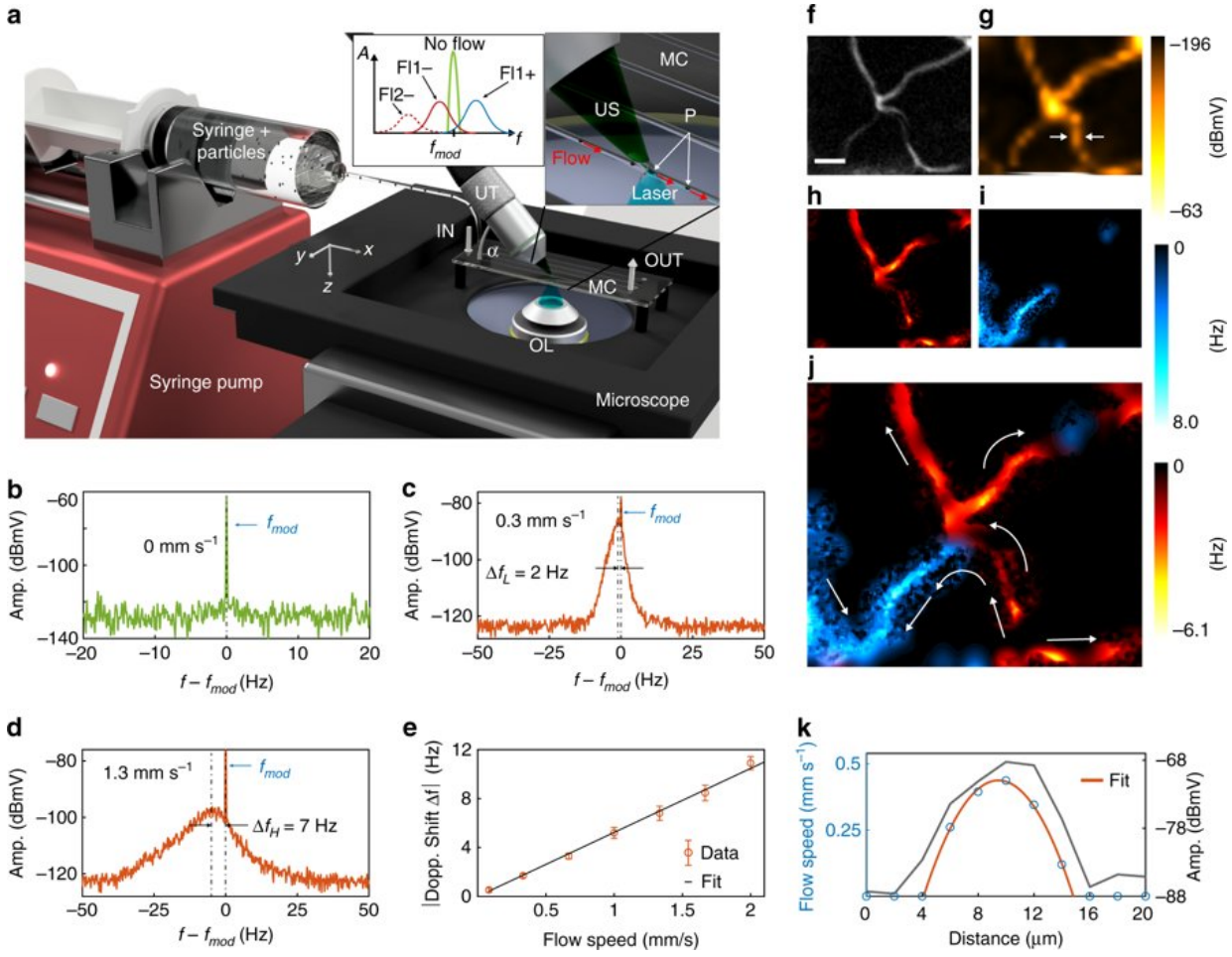
Single- and dual-wavelength FDOM imaging of a mouse ear in vivo. a) FDOM imaging at 488 nm. Cyan color represents the reconstructed image, from nine equally spaced frequencies in the range of 10 to 50 MHz. b–d) Individual images obtained at modulation frequencies of 10, 30, and 50 MHz, which depict the structures in the dashed box in panel a. e) SNR as a function of n frequencies that were used for FSR reconstruction. An asymptotic improvement is observed for n > 8 discrete frequencies. f) A profile view of the dashed box in panel a, which is delineated by a white dashed arrow. It demonstrates the relationship between modulation frequency and imaging resolution. Yellow crosses highlight the imaging resolution as a function of the modulation frequency: faster modulation (50 MHz) can clearly resolve small structures, even down to 4 μm, while slower modulation (10 MHz) cannot. g–l) Hybrid FDOM/multiphoton imaging of a mouse ear following the injection of melanoma cells. g) An overlay image that was obtained using four label-free microscopy modalities: FDOM at 488 nm and 808 nm, SHG at 522 nm, and THG at 348 nm. h) A bright-field image validating the results that were obtained via hybrid microscopy; MC,

melanoma cells. i) FDOM imaging at 488 nm showing vasculature and melanoma cells. j) An FDOM image at 808 nm that shows B16F10 melanoma cells injected in the mouse ear. k) An SHG image showing the collagen distribution in the epidermis. l) A THG image that shows the tissue morphology; predominantly keratinocytes and hair follicles. Credit: *Light: Science & Applications*. Doi: https://doi.org/10.1038/s41377-018-0101-2

For FDOM-based in vivo tissue imaging, the scientists observed the ear of an anesthetized mouse. They obtained artifact-free images with multiple modulation frequencies that matched spatial frequencies of the imaged object. The scientists used a maximum of nine frequencies in the study. The SNR of the image increased from ~14 dB at a single frequency to ~30 dB at nine frequencies for sharper images.

They then observed a mouse ear containing murine metastatic melanoma cells in vivo as before via synchronized excitation of two wavelengths (488 nm and 808 nm) at separate modulation frequencies. Using combined optoacoustic and optical microscopy, Kellnberger and co-workers were able to efficiently image the tissue features (i.e. vasculature, melanoma cells, collagen and keratinocytes) without conventional fluorescent tags or labels.

Kellnberger et al. then performed FD micro-Doppler (μDoppler) measurements with the setup for the first time in a mouse ear for optoacoustic imaging of microcirculatory blood flow in vivo. Before conducting the intended measurements, the scientists used black carbon particles at varying flow rates of circulation in a microfluidic chip to validate the experimental setup. The μDoppler FDOM was employed to generate a map of microcirculation in a mouse ear thereafter. The microcirculatory blood flow revealed gradually increasing speed from the vessel edge to the core.



Optoacoustic imaging of microcirculatory blood flow in a mouse ear in vivo. a A scheme of the μDoppler detection set-up. FL1− flow 1 away from the US sensor, FL2− flow 2 away from the US sensor (FL2−