Fluorescence imaging beyond the ballistic regime by ultrasound-pulse-guided digital phase conjugation

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Fluorescence imaging has revolutionized biomedical research over the past three decades. Its high molecular specificity and unrivalled single-molecule-level sensitivity have enabled breakthroughs in a number of research fields. In vivo applications of its major limitation is its superficial imaging depth, a result of random scattering in biological tissues causing exponential attenuation of the ballistic component of a light wave. Here, we present fluorescence imaging beyond the ballistic regime by combining single-cycle pulsed ultrasound modulation and digital optical phase conjugation. We demonstrate a near-isotropic three-dimensional localized sound–light interaction zone. With the exceptionally high optical gain provided by the digital optical phase conjugation system, we can deliver sufficient optical power to a focus inside highly scattering media for not only fluorescence imaging but also a variety of linear and nonlinear spectroscopy measurements. This technology paves the way for many important applications in both fundamental biology research and clinical studies.

The capability of in vivo fluorescence imaging has expanded rapidly in recent years. Despite improvements in spatial resolution and imaging speed, the achievable imaging depth in live samples has remained very limited, hindering the progress of many research fields. The bottleneck arises from the fact that, to date, only the ballistic component of the light wave has been used for imaging, and this experiences exponential attenuation as a result of random scattering in tissues. Here, we present fluorescence imaging beyond the ballistic regime by combining single-cycle pulsed ultrasound modulation and digital optical phase conjugation (DOPC), and demonstrate a near-isotropic three-dimensional localized sound–light interaction zone. With the exceptionally high optical gain provided by the DOPC system, we can deliver sufficient optical power to a focus inside highly scattering media for not only fluorescence imaging but also for a range of linear and nonlinear spectroscopy measurements.

Controlling wave propagation has been an interesting and important subject in many research fields. In principle, if one can reverse both the propagation direction and the waveform of an optical wave originating from a point (a guide star) inside a turbid medium, one can form an optical focus at the original point, regardless of the thickness of the medium; this process is known as optical phase conjugation (OPC). For imaging, the challenging task is to freely place a guide star at arbitrary locations inside turbid media. Recently, it has been proposed and demonstrated experimentally that a sound wave can be used to modulate light to create a guide star for OPC. As the scattering of sound waves in tissues is negligible compared with that of light, the guide star can be placed at a depth far beyond the ballistic regime of light. However, for practical fluorescence imaging, two challenges remain. First, sound and light are both propagating waves in tissues. Even with focused ultrasound, their volume of interaction is not confined three-dimensionally. Second, given a three-dimensional confined interaction volume, the amount of light that is sound-modulated within a highly scattering medium is very small. Thus, for practical imaging applications in deep tissues we need tremendous optical gain (>10^3) for the phase conjugation beam, and this cannot be provided readily by a conventional phase conjugation system using photorefractive crystals.

Here, we report fluorescence imaging beyond the ballistic regime with a spatial resolution of <40 μm. In contrast to a previous report, we use single-cycle focused ultrasound pulses and tightly synchronized near-infrared laser pulses to achieve a near-isotropic three-dimensional confined interaction volume. The pulsed light and pulsed sound waves are precisely synchronized so that the light wave illuminates the sample only when the single-cycle ultrasound pulse propagates through its spatial focus. Accordingly, the sound modulation zone is confined to <40 μm in the transverse direction by the sound focusing element, and to <40 μm in the axial direction by the temporal profile of the single-cycle sound pulse convolved with the temporal profile of the laser pulse. To provide sufficient and also durable optical power for fluorescence excitation, we used DOPC to perform phase conjugation.

Figure 1a presents a schematic of the operation of the fluorescence imaging system. A high-frequency focused ultrasound transducer launches a single-cycle pulse into the sample. A short laser pulse illuminates the sample only when the sound pulse travels through its focus. The waveform of the frequency-shifted light is recorded by the DOPC system using heterodyne interferometry. To measure the fluorescence signal, the DOPC system sends out the phase conjugation beam, which propagates precisely to the sound focus. A fluorescence detector then measures the power of the emitted fluorescence light. To form a fluorescence image, the entire process is repeated as the acoustic focus is raster-scanned inside the sample. (For a description of the experimental set-up (Fig. 1b), see the Methods.)

Although without wavefront control the input laser light becomes randomized by scattering, it can still excite fluorescence, resulting in background signals. To measure the background level, we translated the DOPC phase pattern by ~30 pixels in both the y- and z-directions on the spatial light modulator (SLM) making the DOPC ineffective (Supplementary Fig. S1a–c). Experimentally, we measured the fluorescence signals with and without translating the phase pattern on the SLM, and the difference between the two signals was used to represent the fluorescence signal at the sound modulation.
position. We define contrast as the ratio of this signal difference to the background signal, which is shown in all the images measured with DOPC.

To measure the point spread function (PSF) of the system, we dispersed 6-μm-diameter fluorescence beads in a 2-mm-thick agar slice and sandwiched the fluorescence agar slice between two 2-mm-thick scattering tissue phantoms (μs = 6.42/μm, g = 0.9306). Details of the phantoms are described in the Supplementary Discussion. Figure 2a,b shows the measured PSF with a sampling step size of 15 μm. The data were resampled with bicubic interpolation, as shown in Fig. 2c,d. Gaussian fittings of the PSF cross-sections (Fig. 2e–g) show that the full-widths at half-maximum (FWHMs) of the PSF are 38.6±2.8 μm, 37.9±2.3 μm and 263±90 μm (±95% confidence bound) along the y-, z- and x-directions, respectively. The achieved focus-to-background ratio (FBR) is ≏3.7 (Supplementary Fig. S1d,e). A similar experiment was also performed with fixed rat brain slices as the scattering media (Supplementary Fig. S2).

To verify that the observed fluorescence signals indeed originated from the ultrasound modulation, we performed a control test by comparing the measurements with and without power on the amplifier for the ultrasound transducer. We sandwiched a 1-mm-thick fluorescence bead agar layer between two 2-mm-thick tissue phantoms (g = 0.9013, μs = 10.5/μm). As shown in Supplementary Fig. S3, the signal was absent with the ultrasound transducer disabled.

To demonstrate the fluorescence imaging capability, we used a glass micropipette to manually create an array of 60-μm-diameter holes, spaced by 120 μm, in a 2-mm-thick agar slice, and injected 6-μm-diameter fluorescence beads inside the holes to create a fluorescence pattern. A direct wide-field fluorescence image is shown in Fig. 3a. The fluorescence hole array was then surrounded by 2-mm-thick tissue phantoms (μs = 6.42/μm, g = 0.9306). Figure 3b presents a fluorescence image of the hole array with tissue phantoms around it. As a result of random scattering, the image diffused to a diameter of ≏2 mm and the structural information was completely lost. We raster-scanned (step size, 30 μm) the position of the acoustic focus and performed DOPC-based fluorescence excitation (raw data shown in Fig. 3c). Two-dimensional Gaussian fitting for each fluorescence hole is shown in Supplementary Fig. S4. We also imaged samples in which the fluorescence features were completely embedded in the middle of a 4-mm-thick scattering medium (g = 0.9013, μs = 7.09/μm; Supplementary Fig. S5).
In our experiments we achieved <40 μm lateral resolution with a near-isotropic three-dimensionally confined modulation zone. The dependence of the modulation zone on experimental parameters is analysed in the Supplementary Discussion. For applications requiring a higher spatial resolution, a higher-frequency ultrasound transducer can be used to shrink the modulation zone. In the fluorescence imaging experiments, we used one-photon fluorescence excitation, for which the fluorescence excitation was not three-dimensionally confined. The background and out-of-focus excitations reduced the achievable signal-to-noise ratio (SNR). However, the background could be dramatically reduced by two-photon excitation at the Ti:sapphire wavelength used in this work. In addition, two-photon excitation can further reduce the size of the PSF by \( \sim \sqrt{N_{\text{mode}}} \) due to the square dependence of the fluorescence excitation on light intensity.

In this Letter, the observed FBR is 1.5–4, a value that needs to be improved for practical imaging applications. Previous studies suggest that the achievable FBR is proportional to \( N_{\text{pixel}} / N_{\text{mode}} \), where \( N_{\text{pixel}} \) is the number of independently controlled phase pixels on the SLM and \( N_{\text{mode}} \) is the number of uncorrelated optical modes at the sound modulation zone. An estimation of the theoretical FBR of our system is presented in Supplementary Discussion. By iteratively focusing light into the sound modulation zone via DOPC, we can potentially achieve a much smaller sound-light interaction volume, leading to better spatial resolution and higher FBR due to the reduced \( N_{\text{mode}} \) (see Supplementary Discussion). Using an SLM with less pixel-to-pixel coupling, higher filling factor and diffraction efficiency, and lower temporal phase fluctuation can potentially improve the FBR by more than one order of magnitude. Moreover, the sound modulation zone can be shrunk by using higher-frequency sound transducers, reducing \( N_{\text{mode}} \) and further improving FBR.

In our experiments, we typically acquired 48–96 interferograms, and the recording time for one DOPC operation was 1.2–2.4 s. We analyse the dependence of the SNR on the camera parameters in the Supplementary Discussion. Using cameras with higher full well charge capacity and frame rate, we can potentially increase the measurement speed by at least one order of magnitude.

For many \textit{in vivo} imaging applications, a transmission configuration is not suitable. However, our technique may be extended to measure sound-encoded backscattered light.

In conclusion, we report fluorescence imaging beyond the ballistic regime with a three-dimensionally confined sound modulation zone, high optical gain and <40 μm lateral resolution in random scattering media. With the capability of focusing sufficient optical power inside random scattering media, our technique can be used not only for fluorescence imaging but also for a variety of linear and nonlinear spectroscopy measurements. It is therefore expected to find numerous important biomedical applications.

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**Figure 2 | PSF measurement.** a. Measured transverse PSF through 2-mm-thick tissue phantoms (\( \mu_s = 6.42 / \text{mm}, g = 0.9306 \)). b. Measured axial PSF. c–d. Corresponding images resampled with bicubic interpolation. e–g. Gaussian fitting of the measured PSF.
**Methods**

**Set-up.** Figure 1b presents the set-up of the fluorescence imaging system. The laser source was a Q-switched green laser pumped Ti:sapphire laser (Photonics Industries) with 778 nm centre wavelength, 10 kHz repetition rate and 20 ns pulse duration. The laser power was controlled by a half wave plate and a polarizing beamsplitter. The laser output was split into two beams, one travelling through a beam expander and entering the DOPC system and, during wavefront recording, serving as the reference beam for interferometry. During fluorescence excitation, this beam illuminated the SLM and became the phase conjugation beam. The other beam was modulated by an acousto-optic modulator (AOM). The frequency-shifted component travelled through a beam expander and illuminated the sample during wavefront recording. The sample was housed inside a water chamber with optical windows, and an ultrasound transducer was mounted on a three-axis translation stage such that the sound wave entered the sample from below. The phase conjugation beam was filtered by a bandpass filter before entering a camera (Andor iXon 3 888 CCD). In most DOPC experiments the camera was used to measure the fluorescence power by summing all of its pixels. There were two exceptions. First, in Supplementary Fig. S1d,e, a wide-field image of a single bead was taken through the clear portion of the sample while it was illuminated with the DOPC system. Second, for the PSF measurement, the fluorescence was recorded from the location of a single bead on the camera while scanning the beam with the ultrasound focus and performing DOPC at each position. The timing and synchronization layout are shown in Supplementary Fig. S6 and the details are discussed below.

**Timing and synchronization.** Supplementary Fig. S6 presents a diagram of the timing and synchronization. A delay generator (DG1, Stanford Research DG645) was used as the master clock for the system. It output a 10 MHz transistor–transistor logic (TTL) pulse train to synchronize an arbitrary waveform generator (AWG, Tektronix AFG3252) and the other delay generator (DG2, Stanford Research DG535). DG1 sent out three 10 kHz pulse trains to trigger the Q-switched laser, the arbitrary wavefront generator and DG2, which controlled the exposure of the CMOS camera. The AWG output a 20 ns single-cycle sinusoidal signal that changed sign with every pulse (Supplementary Fig. S7c) to drive the ultrasound transducer.

The ultrasound frequency was centred at 50,005,000 Hz (50 MHz + 5 kHz) and the pulse repetition rate was 10 kHz. The extra 5 kHz ensured that the residual interferences (aliasing) sampled by any two consecutive laser pulses were out of phase by exactly 180° and were therefore cancelled out by capturing an even number (that is, 100) of laser pulses. The AWG also output a continuous-wave 50,004,990 Hz sinusoidal signal to drive the AOM. The beating between the ultrasound transducer and the AOM was 10 Hz and the CMOS camera ran at 40 Hz, controlled by DG2. For every 25 ms, the CMOS camera allocated the first 10 ms to exposure and the remaining 15 ms to transferring the data to a computer. The driving signals for the ultrasound transducer and the AOM were amplified to 140–160 Vp–p and 30 Vp–p, respectively.

During the editorial process, a similar imaging approach was published28.

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Author contributions

The experiment was designed and implemented by M.C. The fluorescence pattern was created by K.S. The scattering coefficient and the speckle correlation were measured by R.F. All authors contribute to the data analysis and preparation of the manuscript.

Additional information

Supplementary information is available in the online version of the paper. Reprints and permission information is available online at http://www.nature.com/reprints. Correspondence and requests for materials should be addressed to M.C.

Competing financial interests

The authors declare no competing financial interests.