Breaking the spatial resolution barrier via iterative sound-light interaction in deep tissue microscopy

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Optical microscopy has so far been restricted to superficial layers, leaving many important biological questions unanswered. Random scattering causes the ballistic focus, which is conventionally used for image formation, to decay exponentially with depth. Optical imaging beyond the ballistic regime has been demonstrated by hybrid techniques that combine light with the deeper penetration capability of sound waves. Deep inside highly scattering media, the sound focus dimensions restrict the imaging resolutions. Here we show that by iteratively focusing light into an ultrasound focus via phase conjugation, we can fundamentally overcome this resolution barrier in deep tissues and at the same time increase the focus to background ratio. We demonstrate fluorescence microscopy beyond the ballistic regime of light with a threefold improved resolution and a fivefold increase in contrast. This development opens up practical high resolution fluorescence imaging in deep tissues.

Optical microscopy is an invaluable tool in the biological sciences as it enables three-dimensional non-invasive in vivo imaging of the interior of cells and organisms with molecular specificity. Unfortunately optical methods are restricted to an imaging depth of a few scattering mean free path lengths, a severe limitation in many research fields. Recently hybrid techniques that combine the deep penetration capability of sound waves and the molecular contrast of light waves have greatly exceeded the depth limitation of pure optical methods. However, at these extended depths the achievable spatial resolution is restricted by the dimensions of the sound focus. Here we present an approach to fundamentally break the resolution limit of hybrid imaging technologies in deep tissue. Through iterative ultrasound guided optical phase conjugation (OPC), we shrink the sound light interaction volume and obtain a drastically sharper optical focus. This technology paves the way for deep-tissue fluorescence microscopy for biological research and medical applications.

The shallow optical penetration depth has restricted many research fields; it has forced biologists to use transparent model organisms, monolayer cell cultures or histological sections of tissue, just to name a few compromises. Consequently a lot of effort was dedicated to push the depth range in optical imaging and recently substantial progress has been reported using hybrid approaches that combine light and sound. Yet there is still a need for a technique that can take full advantage of the wealth of fluorescent labels and provide microscopic resolution at depths of 1 mm in tissues or deeper. For this goal, we need the ability to focus light tightly beyond the ballistic regime at arbitrary locations.

Recently, light focusing deep inside tissues was achieved using ultrasound guided optical phase conjugation and fluorescence imaging was demonstrated with NIR and visible excitation. An ultrasound focus, which experiences much less scattering than light, is used as a source of frequency shifted light that can be recorded and time-reversed using OPC. Similar to other hybrid techniques, however, the resolving power at large depths is determined by the size of the ultrasound focus, resulting in modest spatial resolutions of 30–50 microns. Further the first demonstrations lacked sufficient contrast for practical biological imaging.

Here we demonstrate fluorescence microscopy beyond the ballistic regime with a lateral resolution of ~12 microns using iterative ultrasound guided digital OPC. We overcome the sound resolution limit by a factor of three and at the same time increase the focus to background ratio (FBR) fivefold. The principle behind our technique can be explained as follows: after traveling through highly scattering media, the incident light field at the ultrasound focus is completely randomized and unfocused. However, if the light was already pre-focused into the ultrasound focus using OPC, a much more confined sound-light interaction would occur.

Let us assume that the transverse profile of the sound modulation zone and hence the phase conjugation beam at the sound focus is defined as $M(y,z)$ and that we employ two digital optical phase conjugation (DOPC)
sound modulated light has a new spatial profile makes the phase conjugation ineffective.

If we assume a Gaussian profile for \( M(y,z) \) and a strong optical focus (large FBR) for a single OPC operation, the transverse FWHM of the PSF decreases as \( 1/\sqrt{N} \). The FBR can be estimated by the ratio of the power of the fluorescence emission for each DOPC excitation was measured and the fluorescent background level was subtracted. The background signal was obtained by lateral translation of the DOPC phase pattern [29,30,31] (30 pixels in \( z \) and \( y \)), which makes the phase conjugation ineffective.

Results

To demonstrate the resolution increase using iterative DOPC, we measured the three-dimensional PSF of our system. To this end, we embedded 6 micron diameter fluorescence beads in a slice of Agar (200 microns thick) and sandwiched the slice between two tissue phantoms (scattering coefficient: 7.63 /mm, g factor: 0.9013) of 2 mm in thickness. The details of the sample preparations are included in the supplementary discussion.

For fluorescence imaging, the ultrasound focus was raster scanned through the sample and at each position iterative DOPC was performed. The power of the fluorescence emission for each DOPC excitation was measured and the fluorescent background level was subtracted. The background signal was obtained by lateral translation of the DOPC phase pattern [29,30,31] (30 pixels in \( z \) and \( y \)), which makes the phase conjugation ineffective.

Discussion

In this study, we break the sound wave limited resolution barrier in the diffusive regime through iterative sound modulated DOPC. This technique improves the resolution in deep tissue fluorescence microscopy towards 10 microns while increasing the focus to background ratio by a factor of 5 at the same time. Better SLM performance such as lower pixel coupling and reduced phase jitter is expected to

![Figure 1](image-url)
improve the current single iteration FBR by more than an order of magnitude, potentially yielding an exact N fold FBR gain through iterations. The increase in sound modulated power allowed us to shorten the acquisition time for the wavefront recording after the first couple of iterations. Moreover, this effect may enable us to focus light even deeper into tissue: by translating the sound focus in small steps between the iterations, the light focus can be gradually guided into deeper regions while maintaining a high sound modulated signal level.

In conclusion, our development is an important step towards practical deep-tissue fluorescence microscopy, providing sufficient resolution and contrast for many applications. Further improvement is expected with two photon fluorescence excitation, potentially leading to sub 10 micron spatial resolution and FBR > 200. We envision that our technique will find numerous applications in neuroscience, optogenetics, medical diagnostics, photodynamic therapy and other fields that require localized light radiation deep inside tissues.

Methods

Setup. Figure 1 e shows our experimental setup: two identical DOPC systems are used either to illuminate the sample with a phase conjugated beam or to record a wavefront emanating from the ultrasound focus within the sample. A Q-switched laser pumped Ti:sapphire oscillator, centered at 778 nm and with 20 ns pulse duration (Photonics Industries, NY), is split into two beams for the two DOPC systems. The two laser beams are used to illuminate the sample via DOPC1 and to serve as a reference beam to record a wavefront on DOPC2 or vice versa. In the beam path of DOPC2, the light is frequency shifted using an acousto-optical modulator such that a 10 Hz beating between the reference beam and the light emanating from the ultrasound guide star results when either DOPC system is used for wavefront recording. This beating is recorded by the camera of either DOPC system, allowing us to recover the wavefront using phase stepping interferometry. Since the laser has a finite coherence length (~1 cm), the path length has to be adjusted depending on which DOPC is used for wavefront recording to ensure proper interference. To this end, the optical path...
length for DOPC1 can be rapidly switched using beamsplitters and two fast mechanical shutters.

The sample is housed in a water chamber with three optical windows. Below the sample, an ultrasound transducer is mounted on a 3-axis motorized stage. Fluorescence emission is filtered by a bandpass filter and is imaged from the side of the sample chamber onto a camera. The camera is not used to record a spatially resolved widefield image but to measure the power of the fluorescence emission by summing all of its pixels. To form a fluorescence image, the ultrasound focus is raster scanned through the sample and at each position, iterative DOPC is applied. For each applied phase conjugation, the fluorescence emission is recorded with the camera. The timing and synchronization scheme was described in a previous publication14.

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Author contributions

The experiment was designed and implemented by M.C. Image data was acquired by M.C. and R.F. The fluorescence pattern was created by K.S. The scattering coefficient was measured by R.F. The numerical simulation was performed by K.S. All authors contributed to the data analysis and the preparation of the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

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