FLUORESCENCE IMAGING

Exploiting sound and noise

A correlation method that combines ultrasound and fluorescence enables imaging in strongly scattering environments.

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Light scattering is a serious impediment to optical imaging in many materials, especially many types of biological tissue. As a result, other modalities, such as ultrasound, which scatters far less, are used for imaging objects deep inside tissue. Still, optical imaging, especially fluorescence imaging, has molecular specificity and sensitivity that is not equalled by other modalities, thanks to the development of sophisticated labelling methods. Hence, there is a great interest in methods that combine the deep-tissue imaging capabilities of ultrasound with the functional imaging capabilities of fluorescence.

Intricate methods have been developed that shape a coherent light beam so that it is guided to a specific location inside the medium by interference[1,2]. A major limitation to applications of coherent light deep in tissue is the short timescale on which the scattering centres inside living tissue move[3,4], meaning that to efficiently direct light toward the desired focus a shaped wavefront must be generated and updated in microseconds.

Writing in *Nature Photonics*, Haowen Ruan and co-workers present a method named fluorescence and ultrasound-modulated light correlation (FLUX), which images the fluorophore density deep inside a medium with ultrasound resolution by using the intrinsic fluctuations of the scattered light[5]. In this method, the random fluctuations are an asset, not a hindrance, which could be of key importance for imaging in dynamic, highly fluctuating environments, such as living animal tissue.

To produce a fluorescence image one needs to detect the fluorescence photons, and know where they are generated. Because the fluorescent light spreads diffusively inside a scattering sample, only very coarse imaging information can be extracted directly from its spatial distribution outside the medium, and the associated resolution rapidly degrades with the depth of the fluorescent object[6]. We can infer where the fluorescence is generated if the excitation intensity is structured in a known way[7].

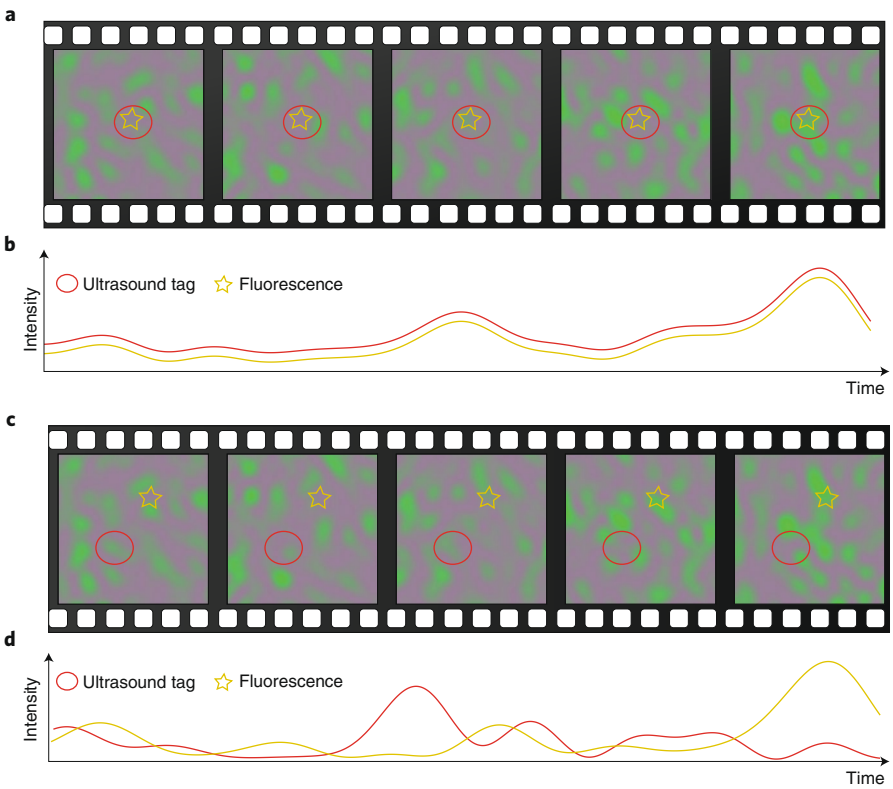

Fig. 1 | Illustration of the principle of FLUX imaging. a, Laser light inside a dynamic scattering medium gives rise to a rapidly fluctuating speckle pattern as depicted by the movie frames. Light in the ultrasound focus (red circle) is tagged by a frequency shift. Light that interacts with the object (yellow star) excites fluorescence. **b**, In the case where the object and the ultrasound focus are at the same position, the intensity of the tagged light and the fluorescence correlate well in time. **c**, When the object and the ultrasound focus are at different positions they see different speckle intensities. **d**, The resulting time traces do not correlate.

If we were able to control the intensity inside the medium, for example by scanning a beam over the object, the intensity-versus-time graph would produce a scanning image directly. However, laser light that is scattered by a static disordered medium forms a high-contrast interference pattern, known as speckle[8], as light arriving through different scattering paths through the medium interferes constructively or destructively. Deep inside a scattering medium the typical size of these speckles is about half a wavelength in each direction.

Let us now consider a single fluorescent particle deep inside the medium, which is illuminated by the excitation laser speckles and emits fluorescent light. Crucially, the total emitted photon flux depends on the intensity of the excitation laser speckle at the position of the particle, which changes rapidly in time due to the motion of the scatterers. This causes the intensity that hits the fluorescent particle to fluctuate

in time, and therefore also of the detected fluorescent photon flux, as depicted in Fig. 1a. Observed over a long enough time, the randomly fluctuating time trace of the intensity is unique for every position inside the medium. However, since the positions of the scatterers in the medium are randomly fluctuating, we don't know which time trace corresponds to each position.

Now with the presence of ultrasound that can penetrate to a depth of several centimetres in some tissue, depending on the frequency, when laser light passes through an ultrasound focus, the carrier frequency of some of the light is shifted by the ultrasound frequency[9]. This is typically less than a millionth of the optical carrier frequency, but enough to enable this 'ultrasound tagged' light to be separated from the untagged light on a detector. The response of a tagged-light detector outside the medium is, therefore, a measure of how much laser power reached the ultrasound focus deep inside the medium, and can be used to very finely characterize light transport in the medium[10].

If the ultrasound focus and the fluorescent emitter are at the same location (Fig. 1a), they experience the same incident intensity fluctuations and as a result the random fluctuations of the fluorescence and tagged light will strongly correlate, as shown in Fig. 1b. If, on the other hand, they are at different locations, the signals fluctuate independently, as depicted in Fig. 1c,d. Therefore, an image can be built up by scanning the ultrasound focus through the region of interest and recording the degree of correlation between the ultrasound-modulated light and the fluorescent light.

Ruan and co-workers have demonstrated FLUX imaging in a proof-of-principle set-up that offers some advantages over living tissue. They used an arrangement of moving diffusers that produces fast-fluctuating speckle patterns, with a tunable fluctuation timescale and speckle size. Moreover, the objects they imaged were very simple, consisting of a few 10-μm-diameter spheres labelled with a strongly fluorescent dye. In the set-up, FLUX imaging was clearly able to locate and discern the spheres when direct observation of the fluorescence offered only a vague diffusive spot.

The approach by Ruan and co-workers has a fascinating relation to 'ghost' imaging, a counterintuitive imaging modality in which the image information is extracted from light that never interacts with the object (for a recent review see ref. [11]). In a typical ghost imaging set-up, a signal beam interacts with the object and is detected without any spatial resolution. At the same time, a position-sensitive sensor detects a reference beam that never interacts with the sample, but is correlated, through quantum or classical[7] correlations, with the signal beam. A computer records simultaneous photodetection events between the two detectors, and produces an image. Quantum, classical and computational ghost imaging experiments have clearly shown the capabilities of this counterintuitive imaging method. However, so far, few demonstrations have been geared towards real-life applications.

In FLUX imaging the fluorescent signal is detected without any spatial resolution, as is the signal beam in ghost imaging. The image is formed by correlation with the spatially resolved ultrasound-modulated light, which is analogous to the reference beam in ghost imaging. Most ghost imaging methods, including FLUX, require no active manipulation of the incident light, making them much more straightforward to implement than imaging modalities that are based on active shaping of the incident wave.

There are, however, some limitations that will likely restrict the cases where the FLUX imaging concept can be effectively used. The most notable seems to be when the volume of the object is much larger than the volume of a single laser speckle. The fluctuations that are essential to form the signal will then be averaged out over many independent speckles. To make things worse, the speckle size and fluctuation timescale in tissue cannot be controlled, and the volume of each speckle is typically much smaller than in the proof-of-principle experiment[5]. This adds up to a substantial reduction of the all-important signal-to-noise ratio and imaging speed, which is why it took Ruan and co-workers several minutes to take each image in their (unoptimized) proof-of-principle experiment. Hence, FLUX imaging in its present form is expected to be useful only in relatively few cases when the object to be imaged is restricted to a small volume, such as tracking a few small fluorescent cells or objects.

A conceptually related method, which was recently demonstrated in vivo, is time-reversed ultrasound-encoded imaging (TRUE)[12,13]. In this method, ultrasound-tagged light is generated inside the sample. Its amplitude and phase are recorded outside the sample, electronically or in a photorefractive material, and subsequently are used to generate a time-reversed wave that is shaped such that constructive interference happens at the ultrasound focus. However, for this time-reverse focusing to work efficiently, the whole record-and-playback operation has to take place within microseconds, as the scattered waves decorrelate strongly on the sub-millisecond timescale[4]. This requires even further speed improvements in hardware. Such speed improvements are physically possible[6] but will need expensive and specialized devices to be developed. FLUX imaging circumvents this problem by employing the fluctuations as its signal generation mechanism[5].

Every method for imaging in scattering media excels at beating some of the many challenges in terms of imaging depth, correlation time, sensitivity, photodamage, resolution and cost, but no one method can overcome all of the challenges yet. The FLUX imaging method overcomes the problem of the rapid speckle fluctuations by using them, making it a promising platform to develop further deep imaging methods that work in dynamic media such as living tissue. ❐

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