Brillouin microscopy – measuring mechanics in biology using light

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Abstract

Mechanical properties of cells and tissues have been shown to play a crucial role in development and disease, but standard techniques for probing them are usually invasive and limited to the sample's surface. In contrast, Brillouin microscopy is an emerging optical technique that enables non-contact measurement of viscoelastic properties of a material with diffraction-limited resolution in 3D. It exploits Brillouin scattering, the interaction between light and acoustic waves intrinsically present in any material due to thermal vibration. Here we introduce the underlying physical principles and discuss applications in biology and medicine.

Introduction - why mechanics matters in biology

Biomechanics, i.e. the study of the mechanical aspects for the structure and function of biological systems, dates back to ancient Greeks: Aristotle, in *De motu animalium*, investigated the locomotion of animals as mechanical systems[1]. More recently, researchers have focused on the biomechanics of the basic elements of biological systems: cells and tissues. One striking example of the importance of mechanical properties in cellular processes came from the seminal work of Engler *et al*, who showed how the stiffness of the substrate influences differentiation of stem cells into the different cell types, thus directing cell fate. In particular, stem cells on a stiffer substrate differentiated into bone cells while stem cells on a softer substrate eventually became neurons[2]. The stiffness of the environment can also affect cell migration, as groups of cells direct their motion towards stiffer substrates, known as durotaxis[3].

These remarkable examples, together with work that showed how cell proliferation, migration and organisation are equally influenced by their and the mechanical properties of the surrounding tissue, gave birth to the field of mechanobiology, which focuses on the investigation of mechanical properties of biological systems on a (sub-)cellular scale, with the aim of better understanding their role in biological processes.

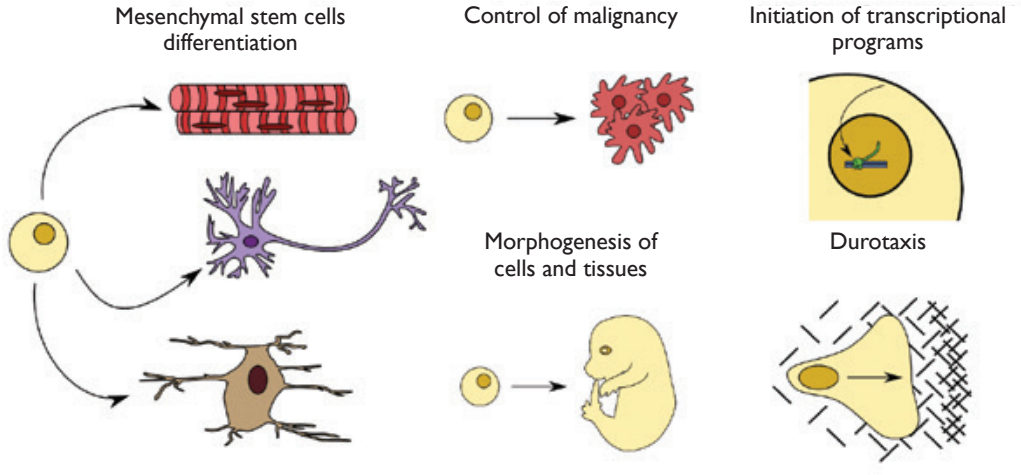

Figure 1. Role of mechanics in biology. Overview of various biological processes where cell and environmental mechanics have been shown to be paramount.

Techniques for measuring mechanical properties

To this end, scientists have been actively developing and employing various tools to quantify the mechanical properties in biological samples.

One of the main techniques in the field is atomic force microscopy (AFM). Here, the sample under investigation is compressed with a calibrated force using a small tip cantilever, and the corresponding deformation is recorded. Then, using a mathematical model based on assumptions about the geometry and homogeneity of the sample, mechanical properties such as Young's modulus can be retrieved, where the Young's modulus is the ratio between stress applied to a sample and strain that it undergoes in response, and hence a proxy for stiffness[7]. Another popular technique in biomechanics is micropipette aspiration. Here, a negative pressure is applied through a narrow pipette which is in direct contact to the surface of a cell and the local deformation of, e.g. the cell surface, is monitored as it is being sucked up into the pipette. This allows to infer properties such as surface tension and contractility[7]. The main limitation of both these techniques is, however, that they require direct contact to the sample, and are thus fundamentally limited to surfaces of cells or tissues.

To access the inside of cells and tissues, techniques have been recently developed that involve injecting micro-beads or lipid-coated droplets inside 3D tissues or large cells to actively apply forces to them with optical tweezers or magnetic fields[8,9]. After calibration, they allow precise measurement of the applied forces and, similarly to AFM, enable to infer Young's modulus from the ratio between the applied force and the observed cellular deformation. This method provides information of stiffness in an *in-situ* context, but requires custom built instrumentation and relies on the invasive introduction of foreign matter into the sample, which might interfere with native local mechanics.

Recently, Brillouin microscopy has emerged as a new technique in the field of mechanobiology to explore the mechanics of living cells and tissues. Originally confined to material science studies, recent developments in spectrometer design are now permitting its use with living samples. Since it is based on light only, it allows to map of viscoelastic properties (i.e. stiffness and viscosity) inside living samples in a non-contact fashion and in three-dimensions with high spatial resolution.

Physical principle of Brillouin scattering

Brillouin microscopy infers viscoelastic properties of a sample indirectly, through the measurement of the speed of thermally-generated sound waves. The technique is named after the French physicist Léon Brillouin (1889-1969) who discovered the interaction between sound waves and light that gives rise to the namesake Brillouin scattering effect[10]. Thermally generated sound waves are intrinsically present in any material at non-zero temperature and do interact with light through a scattering process (see Fig. 2a). This process can most intuitively be described in quantum mechanical terms. Here, in the same way as light can be described as a particle (*'photon'*), a sound wave inside a material can be described as a (quasi-) particle (*'phonon'*). These two (quasi-)particles can in fact collide and thus scatter, whereby in this process their energies and momenta must be conserved. Since this is an inelastic scattering process, a phonon can be either created or destroyed, thus the photon can lose or gain energy. The energy exchange gives rise to two additional peaks in the spectrum of the scattered light (so-called Stokes and anti-Stokes peaks). The difference in frequency between the elastically scattered laser (Rayleigh line) and the Brillouin scattered light, called *'Brillouin shift'*, can be calculated as $\Omega = \frac{2v}{\lambda} n \sin\frac{\theta}{2}$ where v is the speed of sound and

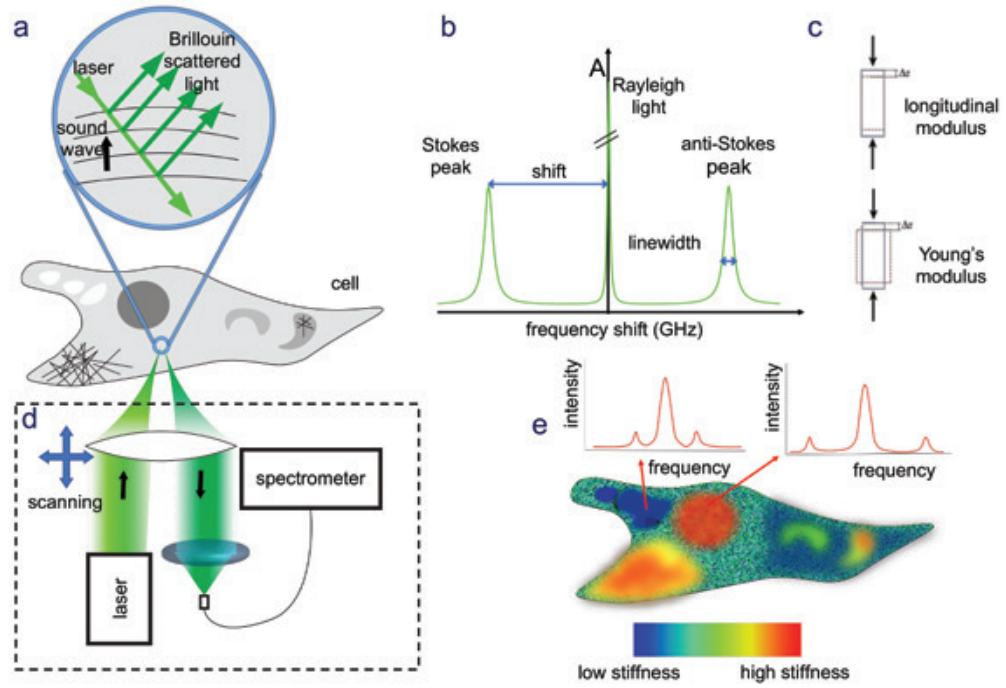

Figure 2. Principles of Brillouin microscopy. a) Laser light interacts with thermally induced sound waves inside the sample. During the interaction energy and momentum are exchanged, resulting in the scattered light to a change direction and frequency. b) The speed of the soundwave and its lifetime determine the frequency shift and the linewidth of Brillouin scattered light, typically in the GHz regime. c) Longitudinal and elastic modulus: both moduli are defined as the ratio between stress applied to the material and the resulting strain. However, in case of the longitudinal modulus the deformation is allowed only in the direction of stress. d) Confocal implementation of Brillouin microscopy: the laser is focused on the sample by an objective lens and Brillouin scattered light is collected by the same lens. Subsequently, a single mode fiber acts as a confocal pinhole and delivers the light to the spectrometer. e) A stiffness map can be constructed by scanning the focal point across the sample and plotting the frequency shift at each position.

θ the angle between incident and scattered light (see Fig. 2b).

Typical frequency shifts for biological samples are on the order of a few GHz. In fact, given the speed of sound in water (~1500 m/s) and assuming visible light (500nm), a typical Brillouin shift Ω for pure water would be ~8 GHz, which corresponds to ~0.008 nm. From this it becomes clear that Brillouin shifts have to be determined with sub-picometer accuracy which poses significant challenges on the spectrometer's optical design.

From sound to mechanical properties

But how is the speed of sound related to mechanical properties? Everyday life experience suggests that the stiffer a material is, the faster the sound travels in it: if you stand close to a railway while a train is approaching, you can hear the rails vibrating long before you can hear the train. In fact, the speed of sound (V) is given by the square root of the longitudinal modulus (*M*) divided by the density (ρ), i.e. $V = \sqrt{M/\rho}$. The longitudinal modulus shares a similar definition with the Young's modulus and is thus a measurement of stiffness. Here, the difference is that, in the former, strain is allowed only in the direction of stress, while in the latter, strain is also allowed in the perpendicular direction of stress (see Fig. 2c). Brillouin scattering essentially probes longitudinal waves, that are "constrained" in the direction of light propagation. Moreover, soundwaves are damped in a viscous material due to energy dissipation, therefore phonons exhibit a finite lifetime. Due to the uncertainty principle, the lifetime of a phonon is inversely proportional to the linewidth of the Brillouin scattered light. As energy dissipation within a fluid is proportional to its viscosity, i.e. a measure of resistance of a material to gradual deformation by shear stress, the linewidth of the Brillouin spectrum can be used to infer it.

Spatial resolution

One of the main advantages of Brillouin scattering is that the volume of the Brillouin interaction, and thus the spatial resolution of the measurement, can be confined to a diffraction limited spot by using a confocal approach. Thus, scattered light coming from out-of-focus regions can be effectively blocked by inserting a pinhole in the detection pathway. Using this approach, diffraction limited resolution (e.g. 0.5μm lateral and 1.5μm axial with 0.8NA) can be achieved in biological samples.

Realisations of Brillouin microscopy

If the scattering phenomenon has been known for almost one century, why is its application in bio-imaging (i.e. Brillouin microscopy) so recent? The reason is mainly technical: to measure pico-meter shifts in frequency, standard grating or prism-based spectrometers are not sufficient. In its early days, Brillouin spectroscopy was mainly applied to questions in solid state physics, using so-called scanning Fabry-Perot spectrometers[11]. This instrument achieves high spectral resolution while effectively rejecting elastically scattered light, which is several orders of magnitude more intense than the Brillouin signal of interest. The major drawback of its design however is its slow speed, as it can take up to several minutes for acquiring a single spectrum. For the investigation of solid materials such as crystals this is not a concern as the samples are usually homogeneous and static. On the other hand, in microscopy thousands of individual measurement points (pixels) are required to construct an image,

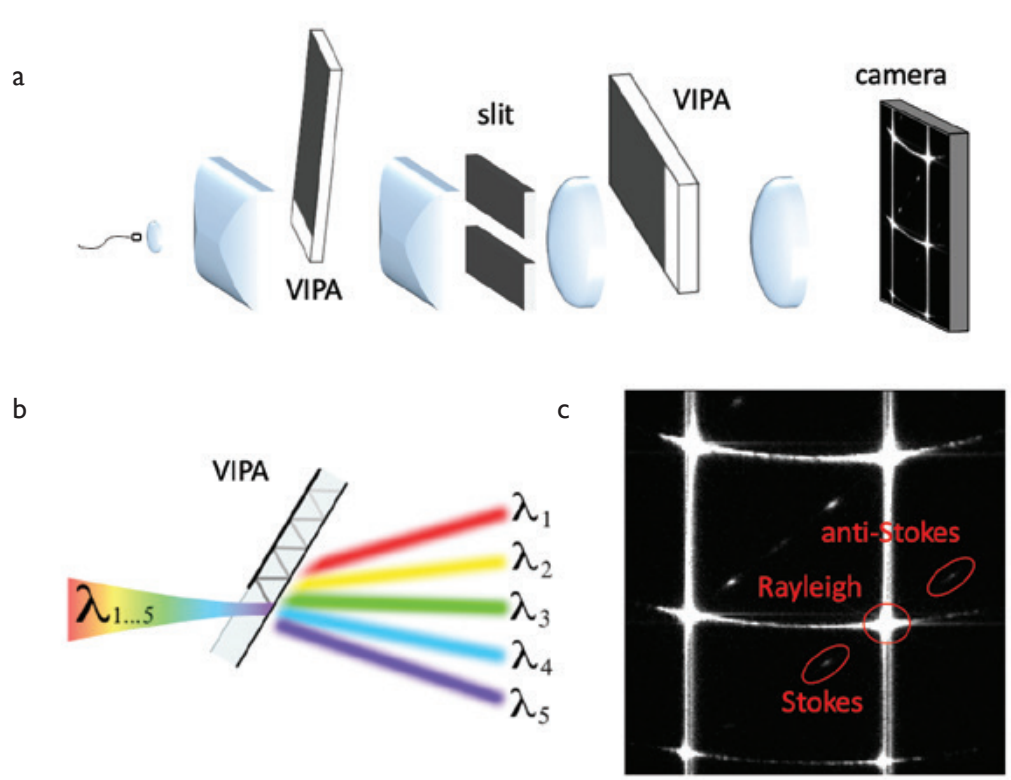

Figure 3. Schematic of a two-stage VIPA spectrometer for Brillouin microscopy. a) The first VIPA generates a diffraction pattern composed of horizontal lines. Subsequently, a slit is used to select one diffraction order and suppress the Rayleigh light. The second stage separates the residual Rayleigh light from the Brillouin signal. b) Working principle of a VIPA: non-monochromatic light is focused on the entrance window and undergoes multiple reflections; interference of these multiple reflections gives rise to angular dispersion (similarly to a diffraction grating). c) Typical dispersion pattern produced by a two-stage VIPA on the camera. Multiple diffraction orders can be seen; each of them shows Stokes and anti-Stokes peaks.

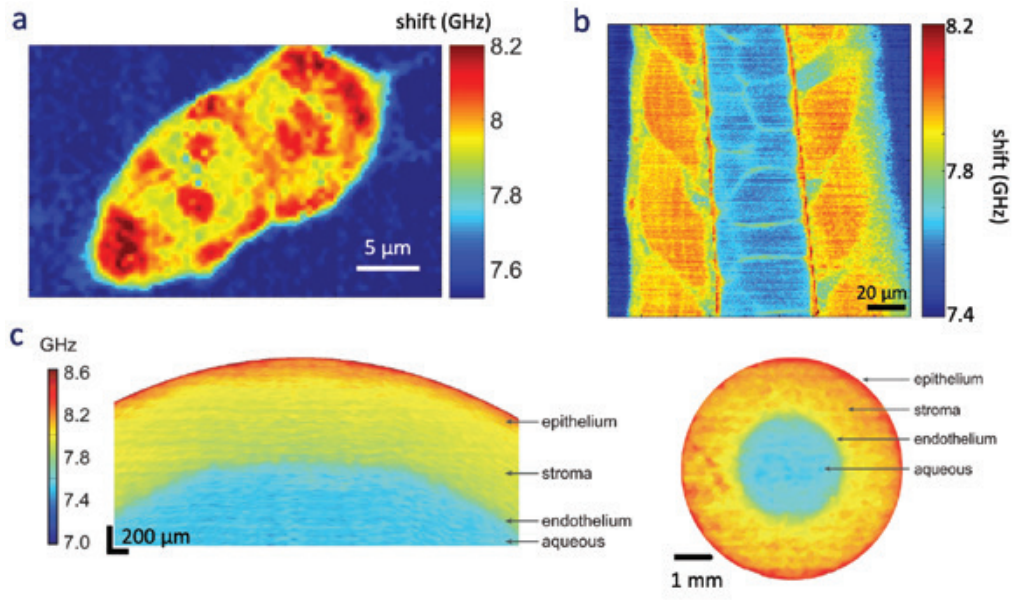

Figure 4. Exemplary Brillouin images of biological samples. a) A mouse embryonic stem cell, showing sub-cellular scale stiffness differences. b) Tail of a zebrafish larva where anatomical structures (muscles, sheath cells, notochord) can be clearly distinguished based on their stiffness properties. c) Left: Sections of a bovine cornea showing the heterogeneity of mechanical properties. Left: Cross section. Right: Optical section from a deep layer. Panel c) adapted from Ref.[17].

resulting in many hours of data acquisition during which the biological sample of interest might change its properties or move. Moreover, prolonged laser light illumination can lead to photodamage in a living sample. Therefore, technical advances were critically required to enable Brillouin spectroscopy in a biological setting. A crucial step in this direction was the introduction of a so-called VIPA-based spectrometer[12] in 2008 (see Fig. 3). The VIPA (virtually-imaged phased array) is an optical device that shares the working principle with a Fabry-Perot: light undergoes multiple reflections inside a resonator defined by two mirrors with engineered reflectivity. At each round trip, part of the light exits the resonator interfering with other multiple reflections. The main difference between a VIPA and a Fabry-Perot is that in the former the light path is designed so that the spectrum is angularly dispersed at the exit (see Fig. 3b). This allows acquisition of an entire spectrum at once using a camera within less than a second, speeding up image acquisition tremendously (~100-fold) compared to traditional Fabry-Perots[12]. To compensate for the less efficient background extinction that a VIPA achieves compared to a Fabry-Perot, two VIPAs with orthogonal dispersion axis can be employed[13]. This allows to spatially separate the Brillouin signal from the elastically scattered light which then can be effectively blocked by a mask (see Fig.3c).

Applications in fundamental research and medicine

As mentioned above, viscoelastic properties play an important role in cellular behaviour, tissue morphogenesis and animal development. Thus, Brillouin microscopy is rapidly finding increasing applications in fundamental biological research (see Fig. 4). Furthermore, it has attracted interest in the medical field as a tool for studying the evolution of certain diseases and for their diagnosis: One of the early applications of Brillouin microscopy in the context of biomedicine was on the human cornea and in particular during the development of keratoconus, a pathological condition that leads to progressive thinning of the cornea[14,15]. It is believed that during its progression the corneal tissue fails to withstand the intraocular pressure, leading to a deformation of the lens. The non-invasive measurement of stiffness of the cornea *in-vivo* is thus essential to understand the development of this pathology and to improve its early diagnosis. Brillouin microscopy represents an ideal tool for this purpose due to its non-invasive nature (see Fig. 4c). Recently, first clinical trials have been started in this direction[16].

Cancer research is another area that might benefit from Brillouin microscopy. Several cancers, and as a typical example breast tumours, are initially detected by palpation when they reach a certain size. The detection of a tumour mass in such a way is due to the higher stiffness of the tissue compared to its environment. This mechanical difference probably also holds true in early stages and assessing it before it can be manually detected may contribute to an early diagnosis. Also, a better understanding of the "mechanical environment", such as reduced viscosity or increased compressibility during cancer evolution can lead to a more effective treatment or to a better monitoring of the response to treatment.

Discussion and outlook

When interpreting Brillouin spectra from biological samples, some important caveats have to be considered. The Longitudinal elastic modulus measured with Brillouin microscopy is different from the Young's modulus that quasi-static techniques (AFM, micropipette, etc.) probe. Since the sound waves probed by Brillouin microscopy are in the GHz frequency range, they correspond to mechanical perturbations on the nanosecond time scale. One obvious question is therefore whether this high-frequency modulus is relevant for biological processes that are typically happening on time scales of seconds-minutes. Recent work showed that there is a correlation between the two regimes and Brillouin microscopy can also be a proxy for low-frequency viscoelasticity[18]. However, such correlation is empirical and sample-dependent, and thus more work is needed to establish a relationship between these regimes. Moreover, a better understanding on the molecular origins of the Brillouin signal is crucial, in order to properly interpret the mechanical measurements obtained. Cells are highly heterogeneous samples, containing tens of different organelles and compartments that all exhibit different mechanical properties on a submicron scale.

Furthermore, in Brillouin microscopy mechanical properties are coupled with optical and material properties (i.e. refractive index n and density ρ). Typically, these properties are estimated or taken from the literature, and thus can introduce errors in the estimation of absolute modulus values, although often investigations are focused on relative changes in mechanical properties only and thus quantitative values are less important. Unfortunately, direct measurements of n and ρ in 3D biological tissues are not straightforward. Several techniques have been proposed for 3D refractive index measurements in biological samples, most notably quantitative phase microscopy[19], although practically they are limited to thin samples such as a single cell layer. Measurement of density appears to be even more challenging but there are empirical equations that relate density and refractive index, making the measurement of refractive index in principle sufficient to retrieve the longitudinal modulus.

In summary, Brillouin microscopy is a technique that allows three-dimensional and non-contact measurements of stiffness and viscosity. Although a solid understanding of the molecular origin of the signal in heterogeneous biological samples is still lacking, recent work points to a great potential both in basic and medical research. Future work will be needed to gain a better understanding of the molecular origin of Brillouin signal and technical advancements will be necessary to improve current microscope performances, in particular increasing the speed (that is still ~2 order of magnitude slower than fluorescence confocal) and reducing photodamage on living samples.

Acknowledgements

We thank D. Richter, J. Czuchnowski and E. Sitarska for help with preparing figures.

References

1. Abernethy, B. *et al. Biophysical foundations of human movement*. (2013).
2. Engler, A. J., Sen, S., Sweeney, H. L. & Discher, D. E. Matrix Elasticity Directs Stem Cell Lineage Specification. *Cell* **126,** 677–689 (2006).
3. Haeger, A., Wolf, K., Zegers, M. M. & Friedl, P. Collective cell migration: guidance principles and hierarchies. *Trends Cell Biol.* **25,** 556–566 (2015).
4. Bosveld, F. & Nodal, T. Mechanical Control of Morphogenesis. *Science (80-.).* **336,** 724–727 (2012).
5. Farge, E. Mechanical induction of Twist in the Drosophila foregut/stomodeal primordium. *Curr. Biol.* **13,** 1365–1377 (2003).
6. Miroshnikova, Y. A. *et al.* Tissue mechanics promote IDH1-dependent HIF1α–tenascin C feedback to regulate glioblastoma aggression. *Nat. Cell Biol.* **18,** 1336–1345 (2016).
7. Diz-Muñoz, A., Weiner, O. D. & Fletcher, D. A. In pursuit of the mechanics that shape cell surfaces. *Nat. Phys.* **14,** 648–652 (2018).
8. Dolega, M. E. *et al.* Cell-like pressure sensors reveal increase of mechanical stress towards the core of multicellular spheroids under compression. *Nat. Commun.* **8,** 1–9 (2017).
9. Campàs, O. *et al.* Quantifying cell-generated mechanical forces within living embryonic tissues. *Nat. Methods* **11,** 183–189 (2014).
10. Brillouin, L. Über die Fortpflanzung des Lichtes in dispergierenden Medien. *Ann. d. Phys.* **44,** 203–240 (1914).
11. Bl/achowicz, T., Bukowski, R. & Kleszczewski, Z. Fabry–Perot interferometer in Brillouin scattering experiments. *Rev. Sci. Instrum.* **67,** 4057–4060 (1996).
12. Scarcelli, G. & Yun, S. H. Confocal Brillouin microscopy for three-dimensional mechanical imaging. *Nat Phot.* **2,** 39–43 (2008).
13. Scarcelli, G. & Yun, S. H. Multistage VIPA etalons for high-extinction parallel Brillouin spectroscopy. *Opt. Express* **19,** 10913 (2011).
14. Scarcelli, G., Kim, P. & Yun, S. H. In vivo measurement of age-related stiffening in the crystalline lens by Brillouin optical microscopy. *Biophys. J.* **101,** 1539–1545 (2011).
15. Scarcelli, G. & Yun, S. H. In vivo Brillouin optical microscopy of the human eye. *Opt. Express* **20,** 9197 (2012).
16. Shao, P. *et al.* Effects of corneal hydration on brillouin microscopy in vivo. *Investig. Ophthalmol. Vis. Sci.* **59,** 3020–3027 (2018).
17. Scarcelli, G., Pineda, R. & Yun, S. H. Brillouin Optical Microscopy for Corneal Biomechanics. *Investig. Opthalmology Vis. Sci.* **53,** 185 (2012).
18. Scarcelli, G. *et al.* Noncontact three-dimensional mapping of intracellular hydromechanical properties by Brillouin microscopy. *Nat. Methods* **12,** 1132–1134 (2015).
19. Jin, D., Zhou, R., Yaqoob, Z. & So, P. T. C. Tomographic phase microscopy: principles and applications in bioimaging [Invited]. *J. Opt. Soc. Am. B* **34,** B64–B77 (2017).

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Carlo Bevilacqua is a shared PhD student between the laboratories of Robert Prevedel and Alba Diz-Muñoz at EMBL Heidelberg.

His main research interest is Brillouin microscopy. He has been working on developing a state-of-the-art, robust, user-friendly Brillouin microscope to help addressing biological question on diverse samples. In parallel, his project is focusing on building a new version of the microscope that tries to tackle some of the current technical limitations.

Carlo studied Physics at the University of Bari (Italy), with specific interest on optics and microscopy.

Dr. Alba Diz-Muñoz is a group leader at the Cell Biology and Biophysics Unit of the European Molecular Biology Laboratory (EMBL) in Heidelberg.

The Diz-Muñoz group studies the crosstalk between mechanical properties and signalling processes that drive morphogenetic processes and fate specification in immune cells, embryonic stem cells and zebrafish embryos. Before that Alba was a joint postdoctoral fellow between the laboratories of Orion Weiner at UCSF and Dan Fletcher at UC Berkeley, where her research focused on understanding how signalling pathways are activated downstream of changes in cell mechanics. In 2011, she obtained her PhD from the MPI-CBG in Dresden, where she worked on cell migration during zebrafish gastrulation between the laboratories of Ewa Paluch and Carl-Phillip Heisenberg.

Dr. Robert Prevedel is a group leader at EMBL Heidelberg.

His primary research interest lies in developing advanced and innovative optical technologies for biomedical imaging and the direct application of these to fundamental and previously inaccessible questions at the forefront of biology. For this, he and his team are drawing from diverse fields such as multi-photon microscopy, active wave-front shaping, photo-acoustics as well as advanced spectroscopy. Robert holds a PhD in experimental physics from the University of Vienna (Austria) for which he developed new approaches for optical quantum computing. During his postdoctoral years, first at the University of Waterloo (Canada) and later at the Institute of Molecular Pathology in Vienna (Austria), Robert worked on innovative optical methods and tools for imaging, with a focus on functional neuroimaging in small model organisms.