Brillouin imaging for studies of micromechanics in biology and biomedicine: from current state-of-the-art to future clinical translation

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Abstract

Brillouin imaging (BI) is increasingly recognized to be a powerful technique that enables non-invasive measurement of the mechanical properties of cells and tissues on a microscopic scale. This provides an unprecedented means for investigating cell mechanobiology, cell-matrix interactions, tissue biomechanics and other fundamental biological questions. Recent advances in optical hardware have accelerated the development of BI techniques, with increasingly finer spectral resolution and more powerful system capabilities. We envision that further developments will enable the translation of BI to clinical applications in disease screening and monitoring. In light of the recent shift towards biomedical applications, this review summarizes the state-of-the-art in Brillouin microscopy and imaging techniques with a specific focus on biological tissue and cell measurements. Key system and operational requirements are discussed to facilitate wider application of BI along with current challenges for clinical translation of the technology for clinical and medical applications.

1. Introduction

Brillouin imaging (BI) is an emerging field that holds the potential to enable unparalleled mechanical mapping of cells, tissues and organs across three dimensions at submicron resolutions [1–3]. Being a label-free and non-contact method to map mechanical properties across three dimensions, BI offers clear advantages over existing methods of characterizing mechanical properties on a micron scale such as atomic force microscopy (nano-indentation), optical tweezers and rheology (‘gold standard’ modes of mechanical characterization such as AFM indentation and rheology are compared against BI in figure 1). Furthermore, BI has an unparalleled spatial resolution (submicron) compared to other acoustic imaging modalities (ultrasound, photo-acoustic or scanning acoustic microscopy [4]). BI techniques are rapidly gaining traction in mechanobiology, chemistry and biomedical science communities as evidenced by a number of specialized reviews published in the last few years [2, 5].

The first Brillouin images of cells were reported in 2015 [6], which inspired a growing number of studies in mechanobiology [5, 7], disease screening [8–10] and cancer research [11] where there is much unknown regarding cell biomechanics-function relationships. The benefits of in vivo BI in application to ophthalmology has already been demonstrated in clinical studies with over 200 patients enrolled to date [12, 13]. In vitro studies have presented compelling evidence towards the benefits of BI for detection of early changes in articular cartilage due to osteoarthritis [14] and potential use of BI as a diagnostics tool in atherosclerotic development of arterial plaques [15]. Together, these preliminary studies strongly support the extension of BI for the study of other diseases in which tissue mechanics are implicated. For example, both systemic fibrosis (an autoimmune disease in which excessive amounts of stiff extracellular matrix accumulate around organs [16]) and endometriosis (where endometrial tissues grow beyond the uterine cavity to form...
fibrotic tissues around organs [17, 18]) are poorly understood or underdiagnosed conditions where new approaches for early diagnosis or treatment are urgently needed.

BI is based on the principles of Brillouin light scattering (BLS), an inelastic scattering of light by thermally-excited pressure waves. The phenomenon has been known since the 1920s due to independent discoveries by Leon Brillouin [19] and Leonid Mandelstam [20]. The original application of BLS was in the material sciences, enabled by the invention of the laser in the 1960s to become an established technique, known as Brillouin spectroscopy, for characterizing condensed matter in solid-state physics, crystallography and geology [21]. BLS in systems of biological significance was first assessed by Randall et al in the late 1970s–early 1980s [22–24]. The last decade has seen a renaissance of Brillouin spectroscopy particularly in biomedical applications [25] due to two factors: (i) the technology required to perform BLS measurements has drastically improved in the last 30 years with the invention and optimization of Sandercok-type interferometer [26], virtually imaged phase array (VIPA) spectrometer [1] and coherent nonlinear detection schemes [10, 27–30], and (ii) the growing field of mechanobiology borne from understanding that mechanical factors are important in the development and functioning of cells and disease pathogenesis [31, 32].

Despite significant improvement in the performance of BI systems and the plethora of applications where this technology can be transformative, BI is currently at a nascent stage that remains within the domain of academic research. Given the increasing interest in BI as a technique for gaining new insights in biology and recognition of its potential for medical imaging and diagnostics, here we provide a comprehensive summary of progress in the application of BI in biological studies for the benefit of interested researchers coming from general biophysics and biomedical backgrounds, without any assumptions of preliminary knowledge of Brillouin light scattering physics. In addition, we classify all existing methods based on Brillouin light scattering and discuss key requirements and challenges that must be overcome in order to enable standardized and widespread clinical application.

2. Overview of Brillouin light scattering phenomenon and methods

2.1. General principles of Brillouin light scattering

Two processes occur when a beam of light passes through matter, namely, absorption and scattering. Atoms and molecules in matter absorb light energy, whereby some is lost as heat and the rest is re-emitted such that the light intensity decays exponentially with the propagation distance. Scattering phenomena depend on the size of a particle in relation to the wavelength of the incident beam and can be classified as either elastic (Rayleigh), where the direction of the beam changes but the frequency of the beam is conserved, and inelastic scattering (Brillouin and Raman), where the frequency and the direction of the beam is altered during propagation [33].

BLS is based on the interaction between photons and acoustic phonons, thermally excited vibrations within a material [19, 20]. As phonons are density perturbations that move at the speed of sound, such an interaction results in a Doppler shift of the scattered light frequency by precisely the phonon frequency Ω. Thus, if the incoming photon has a frequency \( \omega_i \), the scattered photon frequency is given by

\[
\omega_s = \omega_i \pm \Omega.
\]  

The sign ‘±’ suggests that the energy can either be transferred from phonon to photon (‘+’) or vice versa (‘−’).

The phonon frequency \( \Omega \), or so-called Brillouin frequency shift (BFS), is the first measurable output of a BLS experiment. It is directly proportional to the acoustic velocity \( V \) as

\[
\Omega = \pm V q = \pm \frac{4 \pi n}{\lambda} V \sin \frac{\theta}{2},
\]  

where \( q = \frac{4 \pi n}{\lambda} \sin \frac{\theta}{2} \) is the momentum exchanged in the scattering process, \( n \) is the material refractive index, \( \lambda \) is the wavelength of light in vacuum and \( \theta \) is the scattering angle. The acoustic velocity, in turn, is the function of material density \( \rho \) and the longitudinal modulus \( M \)

\[
V = \sqrt{\frac{M}{\rho}}.
\]  

Equations (2) and (3) suggest that, in the assumption of constant \( n \) and \( \rho \), the data collected from BLS experiments can be used to assess \( M \)—one of the elastic moduli that is defined as the ratio of axial stress to axial strain in a uniaxial strain state. \( M \) should not be confused with Young’s modulus \( E \). The main difference between the two moduli definitions is in the boundary conditions: in the deformation defining \( E \), the object
is allowed to expand or contract in directions orthogonal to the direction of applied stress with conservation of volume. The deformation associated with the longitudinal modulus $M$, in contrast, has fixed boundaries in the orthogonal directions to stress application, i.e. the volume and density of the object changes. This difference is clearly represented in the two common mechanical tests, confined and unconfined compression, which allow the measurement of $M$ and $E$, respectively. Interestingly, the difference between the longitudinal and Young’s moduli becomes particularly important in soft matter and specifically, in hydrated biomaterials where $E$ and $M$ differ by several orders of magnitude and are not always correlated [34]. This has been previously discussed in a number of seminal works [2, 35].

BLS measurements involve collection of spectral data, a schematic example of water and hydrogel spectra are illustrated in figures 2(A) and (B). The spectra are centered on the frequency of the probing laser and consist of a number of peaks: a Rayleigh peak (R) at zero frequency and a Brillouin doublet, Stokes (S) and anti-Stokes (AS) peaks at frequencies given by equation (2). For most biological materials, $\Omega$ is within the range of 1–10 GHz (for a visible laser probe) and it can be significantly higher in solid-state materials.

The second measurable output of a BLS experiment is the full width at half maximum (FWHM) of the Stokes and anti-Stokes peaks, $\Gamma$. This parameter is inversely proportional to the acoustic lifetime $\tau \sim 1/\Gamma$, i.e. materials with larger acoustic damping show broader Brillouin peaks.

The exact values of $\Omega$ and $\Gamma$ are determined not only by the frequency and lifetime of phonons traveling inside the material, but also by the scattering geometry, ambient temperature, properties of the imaging objective lens, and the sample’s internal architecture. Overall, there are three spatial scales to consider: (i) the phonon length, (ii) phonon propagation distance and (iii) the imaging volume [36]. Depending on these three parameters, different effects can be observed. For example, if the internal structure of a multicomponent material has spatial scale much smaller than the phonon length, the frequency of an acoustic phonon and its lifetime are represented by the average properties across the imaging volume.

Consider a hydrogel material illustrated in figure 2(B): the dimension of polymer fibers that construct solid network of hydrogel is on the order of 20–50 nm, whereas the phonon length is 200–300 nm and the imaging volume is typically 1–10 $\mu$m$^3$. The Stokes and anti-Stokes peaks measured in this scenario are broadened due to inhomogeneity and viscosity of the gel, and shifted toward higher frequency ranges (figure 2(B)) compared to the Brillouin scattering signal of pure liquids such as water (figure 2(A)). This oversimplified example is only for demonstrating and explaining the general features of BLS measurement and data. In general, interpretation of $\Omega$ and $\Gamma$ is not a trivial matter and largely depends on the type of material, internal structure and the mathematical model(s) adopted. More commonly applied models are presented and discussed in section 5.

2.2. Terminology of methods based on Brillouin light scattering

As BI is becoming more established with more multidisciplinary researchers joining the field, there is a compelling need for unity regarding the use of terminology and clarity between different concepts, methods, technologies and descriptions of Brillouin applications that are often used interchangeably and/or mixed in the current literature. To address this need, we identify and define current techniques and technologies in the
Figure 2. Schematic representation of the interaction between light and sound in media of varying complexity: (A) water and (B) hydrogel. The Stokes (S) and anti-Stokes (AS) peaks measured in water have smaller full width at half maximum (ΓW) and are positioned closer to the central Rayleigh (R) peak. Broadening (ΓW < Γh) and spectral shift of Stokes and anti-Stokes peaks in hydrogel (ΩW < Ωh) reflect the multicomponent and viscoelastic nature of hydrogel consisting of a polymer fiber network immersed in liquid.

field and propose their classification under the umbrella term of Brillouin imaging for clarity. Our objective is to provide a guide to BI terminology which can serve as guidance for the growing Brillouin community.

Brillouin imaging is an umbrella term that encompasses all methods that employ BLS as a contrast mechanism to generate an image or ‘map’ of the sample of interest, whether by spectroscopy, microscopy or a combination of various techniques. We encourage adoption of this term whenever the technical details of a method are secondary in significance to the goal of a narrative and elaborate with the appropriate technique.

Brillouin spectroscopy refers to the single point detection and analysis of Brillouin light scattering collected from a material of interest. The size of the scattering volume is not restricted in this application and is mostly chosen based on considerations related to the structure of the material and scattering geometry.

Brillouin micro-spectroscopy refers to the detection and analysis of a Brillouin light scattering signal from a micro-sized volume of a material. This typically requires implementation of high-numerical aperture lenses that can lead to spectral broadening and distortions of peaks in the scattered light spectrum [37].

Brillouin microscopy (BM) generally refers to the combination of Brillouin spectroscopy with confocal microscopy. Due to the configuration of confocal microscopy systems, which typically operate via epi-illumination, only scattered light propagating in the backwards direction is collected and passed through the microscope aperture. The spectroscopic analysis is then performed for each spatial location within a 3D sample by scanning the object mounted on a 3-axis microscopy stage [3].

Shear Brillouin light scattering microscopy is a method adopted for mapping both longitudinal and shear elastic moduli within a 3D sample. A shear BLS scattering instrument strongly resembles a standard confocal Brillouin microscope, but the probing light is sent at an angle to the optical axis of the objective lens and enters the lens at the periphery [38]. The scattered light collected by the same lens consequently has two components that come from interaction with the longitudinal and the shear pressure waves, resulting in respective retrieval of longitudinal and shear elasticity metrics.

Line-scanning Brillouin microscopy refers to a sub-division of Brillouin microscopy in which the signal is collected instantaneously from an entire line inside the sample, compared to point-by-point acquisition [39].

Stimulated Brillouin scattering (SBS) microscopy is the term adopted for a Brillouin microscopy method based on the SBS process in which inelastic scattering of the probe laser is enhanced by the addition of a pump laser. SBS experiments are typically based on coherent heterodyne detection using radio-frequency modulation formats, which improves scattering efficiency and enables faster acquisition time [27, 28].

Impulsive Brillouin scattering (ISBS) microscopy is an alternative to SBS and employs two crossing pump beams to generate a transient density grating at the point of intersection within the sample, whereby
scattering from this grating is measured with a continuous wave probing laser beam \[29, 30\]. This modulation is recorded in the time domain and the phonon frequency associated with the modulation is extracted through Fourier transformation. Despite the complexity of the experimental setup, the technique offers the clear advantage of time-resolved spectroscopy. The latter feature has been acknowledged by an alternative name of ISBS, i.e. Brillouin Imaging via Time-Resolved Optical Measurements (BISTRO) \[10\].

Brillouin endoscopy is an approach to Brillouin imaging based on the integration of a BLS spectrometer with a fiber optic probe for flexible and in-situ collection of the scattered light \[40\].

### 3. Technology and system requirements for BLS-based biological imaging

#### 3.1. Brillouin hardware

A Brillouin imaging setup consists of several fundamental components including: (i) a laser, (ii) an imaging system, and (iii) a spectrometer or spectral detection instrumentation (figure 3(A)). The challenge in building such a system comes down to inefficiency of the spontaneous BLS process (1 photon in a billion) and a narrow range of frequencies detected in BLS experiments (0.1–20 GHz). Typical optical spectrometers based on prisms and gratings fail to provide sufficient spectral resolution. Specialized instruments designed for spectroscopic measurements in the MHz range, e.g. tandem Fabry–Perot interferometers or lock-in detection schemes common in microwave and radio-frequency physics are better suited for Brillouin spectroscopy applications. Long phonon lifetimes (microseconds) require frequency-stabilized, high-coherence light sources to probe photon-phonon interactions. Special filters may be necessary to remove unwanted background composed of stray light, Rayleigh scattered light, Raman and fluorescence signals \[41, 42\]. A scientific grade camera or a photo-multiplying tube should be used to capture the relatively low number of backscattered photons generated in BLS. In addition, electron-multiplying sensors (EMCCD cameras) are sometimes necessary to achieve sufficient measurement sensitivity, especially for imaging in turbid media. Each component of a Brillouin imaging system is discussed in detail in the following section.

#### 3.1.1. Laser

The spectral range of Brillouin light scattering (typically between 1–20 GHz and sub-GHz line widths), determines the choice of laser suitable for intended experiments. The spectral width of the laser emission should be well below the Brillouin linewidth (~1 MHz or less) to avoid spectral broadening of Brillouin peaks and maximize inelastic scattering. The frequency stability and noise of the laser are equally important for reliable detection of Brillouin signals and sufficient signal-to-noise ratio. Frequency locking to an external reference is often required to reduce temporal drifts of the laser frequency due to temperature fluctuations \[25, 43\].

Apart from the above considerations, the ultimate choice of the laser source is also determined by sample transparency. Brillouin imaging and microscopy of biological materials are ideally performed within the ‘tissue window’ i.e. the region of minimal light absorption and scattering of biological cells and tissue, which occurs between the mid-visible to near-infrared regions \[44\]. The UV and blue/green spectral regions are not suitable for BI since light within this range of wavelengths can be absorbed by DNA, melanin, fat, bilirubin, or beta-carotene. In the infrared region, water absorption becomes dominant and therefore lasers emitting these wavelengths are also not ideal for BI. On the other hand, BLS is a dipole-radiative process, thus the
scattering efficiency is proportional to $\lambda^{-4}$, so the signal intensity is significantly weaker at long wavelengths.

Another important advantage of using shorter wavelengths for BI is the ability to achieve higher spatial resolution (proportional to $\lambda$).

Brillouin studies so far have used frequency doubled solid-state lasers, most using 532 nm wavelength of Nd:YAG laser [3, 6, 38], some using 561 nm [7, 14, 15, 34] and two using 671 nm [40, 41]. For in vivo studies, where there is a risk of photodamage to light sensitive samples, BLS studies have used near-infrared wavelength (780 nm semiconductor lasers and additional spectral purification elements [9, 27, 45, 46]. A recent study by Nicolic et al [47] showed that the use of 660 nm wavelength enables live cells to be irradiated with 82 times more energy than at 532 nm, thus shortening the acquisition time and allowing approximately 34 times higher signal-to-noise ratio. Clear advantages are that measurements can be performed over longer periods of time with lower risk of photodamage, over larger fields of view with shorter point-to-point steps, and with improved precision.

3.1.2. Spectrometer

The biggest challenge of Brillouin imaging has been in achieving sufficient spectral resolution and sensitivity necessary to resolve a relatively weak Brillouin signal only a few GHz away from the frequency of the laser. Several strategies and spectrometer designs have been suggested to address this challenge, all of which will be discussed in the following section.

3.1.2.1. Tandem Fabry–Perot interferometer

Spectroscopic measurements with MHz resolution have traditionally involved scanning interferometers, e.g. Fabry–Perot interferometers (FPI) [26]. The main optical element of an FPI is a set of two parallel mirrors separated by a free space of a specified length, or so-called Fabry–Perot etalon. Depending on the distance between the mirrors, light travels through or is reflected by the instrument. By scanning the distance between the mirrors, the spectral selectivity is achieved within the free spectral range of the instrument, which is the spectral distance between neighboring maxima in the transmission spectrum. A standard FPI has one etalon in its construction and it can be set up to have required resolution in the range of a few tens of megahertz, which is not always sufficient to detect Brillouin signals in semi-opaque and turbid samples. The coupling of two synchronized Fabry–Perot interferometers results not only in increased contrast, but also helps to broaden free-spectral range of the instrument [48].

A modern tandem Fabry–Perot interferometer (TFPI) can achieve contrast of up to 150 dB and hence is ideally suited for Brillouin imaging of turbid samples. Originally designed by J. R. Sandercock in 1970s, the TFPI was recently improved by the addition of optical isolators, which provide an extra 50 dB of optical contrast compared to the original design [36, 49]. FPIs are versatile instruments with several adjustable parameters. For instance, the mirror spacing can be changed from 0.1 mm to 30 mm to tune spectral range and resolution. Thus the same instrument can be used for Brillouin measurements of solid samples like quartz ($\Omega \sim 30$ GHz) and soft samples such as hydrogels ($\Omega \sim 6$ GHz at the laser wavelength of $\lambda \sim 600$ nm) with equal resolving power.

The TFPI was commercialized by Table Stable Ltd, which has contributed to its popularity for Brillouin spectroscopy and the spread of technology in recent years. The downside of a TFPI system is the relatively long signal acquisition time (typically 1 s or more). The minimum acquisition time is limited by the scanning speed of the mirrors and cannot be significantly improved. Although acceptable for single point measurement, long signal acquisition times are prohibitive for scanning across 3D volumes; a high number of sampling points (>10 000) can take many hours, which is not suitable for live cell imaging.

3.1.2.2. Virtually imaged phase array spectrometer

Initially introduced in telecommunications [50], a virtually imaged phased array (VIPA) can be used to spatially spread the frequencies of the scattered beam. This constitutes the second, non-scanning, type of spectrometer for imaging based on spontaneous BLS [1]. Single-stage and multi-stage VIPA spectrometers are both popular options for Brillouin measurements that require faster acquisition speeds than possible with TFPIs [8, 51]. A simple design of VIPA optical element, consisting of a glass etalon with reflection coatings and a transparent input window [50], offers sufficiently large angular dispersion to separate Brillouin Stokes and anti-Stokes peaks from the Rayleigh signal. The resolution of VIPA spectrometer is in the order of ~0.5–1 GHz and is limited by the fabrication tolerances of the etalon. Typically, this is not enough to resolve FWHM of Brillouin peaks (50–500 MHz) and the measured signal represents a convolution of the material linewidth and the instrument response function. This is a significant downside of VIPA-based Brillouin spectrometer, as only the measurement of the BFS is permitted. This spectrometer has no movable parts and no need for synchronization, making it much cheaper than TFPI and significantly easier to install and operate.
A typical acquisition time of VIPA spectrometers is on the order of 100 milliseconds, depending on the sample transparency and laser power, which is at least an order of magnitude faster than TFPIs. The contrast available with single-stage VIPA spectrometer is, however, only 30 dB, making a single-stage system unsuitable for measuring turbid samples. Addition of the second VIPA etalon, aligned at 90° to the first, can boost the contrast of the spectrometer to 50–60 dB but with a reduction in signal strength. Overall, VIPA spectrometers are a good choice for transparent samples and quick measurements which aim to detect relative changes in the Brillouin frequency shift without a need for detailed spectra. The moderate cost of this system also suggests that it can be suitable for routine monitoring and point-of-care diagnostic environments. It follows that the only clinical trials to date (diagnostics of ocular health) are based on Brillouin imaging using a VIPA-type spectrometer [9]. Recently, a VIPA-based spectrometer that includes a tunable laser line cleaning filter to boost SNR to 60–65 dB has been commercialized by LightMachinery.

3.1.2.3. Coherent nonlinear techniques
Nonlinear techniques such as stimulated Brillouin scattering (SBS) imaging [27, 28] and impulsive Brillouin imaging [29, 30] form an alternative group of Brillouin imaging methods that exploit the formation of an acoustic wave by electrostriction or thermal excitation. The phenomenon of electrostriction is associated with the change in material density as the result of application of a strong electromagnetic field. Light absorption and thermal expansion could be the second route for excitation of phonons in the material. Regardless of mechanism, phonons generated as the result of exposure to high-intensity periodic optical fields can be probed by a weaker beam of light to obtain the phonon propagation speed and ultimately some mechanical properties of a material. The efficiency of phonon generation in this case is proportional to the pump light intensity, and hence the probe scattering could be orders of magnitude stronger than in the scenario of spontaneous Brillouin scattering. High scattered signal magnitude translates into better signal-to-noise ratio of stimulated versus the spontaneous techniques, and consequently faster acquisition times.

Stimulated Brillouin imaging utilizes a strong pump and a weak probe beam, which are slightly detuned in frequency. By scanning the probe frequency, phonon generation reaches the maximum efficiency whenever the frequency detuning between pump and probe coincides with the acoustic resonance, which corresponds to the frequency of one of the acoustic modes supported by the geometry and composition of the sample. The detection of the scattered signal is achieved via radio-frequency modulation formats and lock-in detection schemes [27]. Such schemes are better suited for resolving GHz frequency shifts with resolution only limited by the laser bandwidth (that can be below 1 MHz).

In the impulsive Brillouin imaging technique, the acoustic wave is formed by crossing high-power pump beams at the same frequency. The density grating built by electrostriction and thermal excitation then propagates through the samples and is probed by a weak probe beam (figure 4(A)). The scattered signal is detected via a fast photo-diode and the frequency is extracted by fast Fourier transform [29, 30]. This scheme enables assessment of the time-dependent dynamic processes, e.g. polymerization processes or environmental changes on millisecond time scales, due to the fast processing times and direct access to temporal characterization of the travelling acoustic waves (see schematic of the measurement system in figure 4(B)). The key parameters of different types of Brillouin imaging techniques are summarised in table 1.

3.1.2.4. Picosecond ultrasonics for time-resolved mechanical imaging
Picosecond ultrasonics (PU) is another branch of Brillouin spectroscopy that utilizes GHz Brillouin oscillations in the time domain, which enables time-resolved imaging of biological structures on the cellular and sub-cellular scales [4, 55, 56]. The readout of Brillouin frequency and acoustic attenuation in PU is very similar to the impulsive Brillouin imaging technique, i.e. by scattering a probe wave off the propagating pressure pulses. However, the excitation of the pressure pulses is distinctly different to the rest of Brillouin imaging methods because excitation is obtained by pumping ultra-short pulses in a layer of a metallic material to generate acoustic waves within the layer. Imaging living cells requires an optimized transducer design to avoid heating and photon damage of cells [4, 56]. Despite the complexity of the measurement apparatus and sample preparation limitations (cells need to be extracted from their environment and positioned on the specially designed transducer), the performance characteristics of such an imaging system are similar to more conventional Brillouin imaging techniques with the acquisition time per point being on the order of 1 s to reach reasonable SNR ratio [4] and spatial imaging resolution of 1 µm [4, 56].

3.1.3. Imaging system
3.1.3.1. Confocal microscope
Koski and Yarger were the first to combine Brillouin spectroscopy with confocal microscopy to achieve Brillouin imaging of heterogeneous samples [1]. The spatial resolution of this experiment (20 µm) was far from modern standards in confocal imaging, but later studies pushed the spatial resolution to the diffraction
Table 1. Key parameters of Brillouin imaging techniques (SI unit abbreviations).

| BI Technique | BLS Type | Acquisition time (s) | Spectral resolution (MHz) | Contrast (dB) | Sample suitability | Power* (mW) | Refer. |
|--------------|----------|----------------------|----------------------------|---------------|---------------------|-------------|--------|
| VIPA         | Spont.   | 0.1–10               | 500                        | 10–30 (1 stage) 30–50 (2 stage) | Liquids, hydrogels, cells, tissues, organs | 1–30        | [3, 6, 7, 14, 43, 51, 52] |
| TFPI         | Spont.   | 1–100                | 10–100                     | 100–150       | Liquids, cells, tissues | 10–30      | [2, 11, 36, 43, 49, 53, 54] |
| SBS          | Stimul.  | 0.05–10              | 4                          | 20–50         | water, intralipid    | 270 mW (pump) | [27, 28] |
| ISBS         | Stimul.  | 0.001–0.05           | 1                          | 10            | Methanol, NaCl solution, hydrogels | 20 mW (probe) 4–40 µJ (pump) | [10, 29, 30] |

* on sample
limit and achieved subcellular imaging with a beam focus to dimensions below 500 nm [6, 7]. The epi-illumination principle common to confocal imaging, i.e. the objective used to illuminate the sample, serves for light delivery and collection (figures 3(A) and (B)). The measurement is set up in a reflection mode and is suitable for 3D bulky samples and in situ imaging. However, the 180° scattering geometry restricts phase-matching between optical and acoustic waves, enabling only one type of interaction with longitudinal acoustic waves [3]. Thus, the information available in confocal imaging experiments is limited to the measurement of the longitudinal acoustic velocity and the lifetime of longitudinal phonons. Full characterization of mechanical properties necessitates other types of mechanical perturbations e.g. shear and Young’s moduli.

3.1.3.2. Platelet scattering configuration
Platelet scattering geometry enables the measurement of acoustic velocity anisotropy by changing the angle of incidence, and is suitable for characterizing mechanical properties in tissues with complex structure and directions of symmetry. In a platelet scattering configuration, a thin section of the sample (100–200 µm) is positioned on a reflecting surface (a mirror). An incident light beam probes the specimen at a non-zero angle to the normal of surface of the specimen, while the mirror holding the sample is rotated 360° around the normal. This scattering configuration allows access to the velocity of two types of acoustic waves: those parallel and perpendicular to the mirror surface. Platelet scattering configurations have been widely used to study anisotropy of elastic properties in collagen and elastin fibers [53], cartilage [57] and bone specimens [58].

3.1.3.3. Fiber-integrated probes
The non-contact and label-free nature of Brillouin imaging makes this technology an ideal solution for in vivo and in situ imaging for a number of applications in biomedical or industrial monitoring. The footprint of a Brillouin imaging system and its reliance on bulk-optical alignment is, however, the major drawback in the translation of this technology into real-world environments. Despite the dramatic improvement in the performance of Brillouin microscopes since the first publication by Koski and Yarger in 2005 [1], much more effort is needed to integrate the instrument into a scalable, robust and easy to transport...
device. Some progress in this objective was made by Kabakova et al with a study of the performance of Brillouin fiber probes [40]. Single and dual fiber probe designs were evaluated in terms of their collection efficiency and imaging performance, with dual fiber design offering a straightforward solution to the removal of unwanted fiber-generated Brillouin scattering background [40]. However, further work is needed to improve the efficiency of the scattered light collection in such fiber probes and to create monolithic fiber devices capable of 360° imaging within the tissue [59].

3.2. Multimodal and correlative systems
Application of BLS as a contrast mechanism to confocal imaging of biological samples by Scarcelli et al technically represents the first combinative Brillouin microscopy system. Currently, systems that combine Brillouin microscopy with other established techniques are rapidly emerging, capable of providing specific quantitative analyses of the chemical and physical properties of samples with simultaneous viscoelastic mapping inferred from Brillouin imaging. These systems represent an important advancement in correlative microstructural characterization. By allowing direct correlation of distinct metrics, more holistic characterization and elucidation of biological phenomena can be achieved.

3.2.1. Brillouin-Raman microscopy
Raman spectroscopy is an established technique for probing the molecular structure, composition and vibrational modes of compounds. Based on inelastic scattering, it can provide a unique ‘fingerprint’ that can be used for chemical identification. Recent advancements have enabled the integration of what were previously standalone systems to provide simultaneous Brillouin and Raman light scattering detection capable of correlating viscoelastic characteristics with chemical specificity (figure 3(C)). As cellular processes are governed by a plethora of complex chemical interactions, Brillouin–Raman systems hold significant potential for studying biological processes such as tissue remodeling in disease progression and wound healing, or cellular reactions to physico-chemical changes e.g. drug screening. The capacity to correlate chemical specificity to viscoelastic modulus is also important for the characterization of hydrogel-based tissue engineering substrates where stiffness is determined by the degree of crosslinking and hydration.

Higher contrast and subcellular resolution imaging with chemical and mechanical specificity was first reported by Palombo et al to site-match Brillouin maps of ex vivo Barrett's esophagus tissue with chemical signatures by Raman and FTIR [60]. Measurements were initially performed separately and thus required significant efforts in image processing and data analysis due to ambiguity in physical location identification and matching between the three sets of experiments. High-performance simultaneous Brillouin and Raman measurements, collected from the same imaging volume within the sample, was later achieved by the same group [36, 49, 61]; this represented a dramatic improvement in imaging and data post-processing time, as well as significantly simplified results analysis and interpretation.

3.2.2. Brillouin-fluorescence microscopy
Elsayad et al engineered a microscope system that integrated fluorescence emission detection with detection of BLS and called the method fluorescence emission–Brillouin scattering imaging (FBi) [62]. The authors demonstrated that this approach can be used to investigate regulatory events that alter cellular and extracellular mechanical properties of living cells within tissues. This work also revealed that the cytoplasm near the plant cell membrane and the extracellular matrix are localized regions of higher stiffness and the stiffness varies along and perpendicular to the cell growth axis [62].

Another study integrated Brillouin micro-spectroscopy with microfluidics to achieve phenotyping of the cell nucleus at a throughput of 200 cells per hour [8]. Fluorescence imaging in this study was used to verify Brillouin signatures and separate signals associated with nuclei and cytoplasm.

Finally, the combination of Brillouin and fluorescence imaging was used to study the stiffness evolution and growth dynamics in active, live aeruginosa biofilms [70]. The spatial distribution of the fluorescence signal in this study helped to correlate the value of the Brillouin frequency shift with the mass distribution of live bacteria within the colony.

3.2.3. Brillouin imaging and optical coherence tomography
Brillouin imaging and optical coherence tomography (OCT) was recently combined to study tissue biomechanics during cranial neural tube closure in mouse embryo development in situ [71]. OCT, an optical imaging technique that also does not require physical contact or labels, provides structural information, whereas the mechanical contrast supplied by Brillouin imaging at submicron resolution showed gradients in the longitudinal modulus across the cranial tube and tube stiffening at later stages of embryo development [71].
3.3. Sample preparation

A key limitation for optical microscopy is that biological samples beyond a thickness of several cells tend to be opaque due to the cellular and inhomogeneous structure of tissues. As Brillouin spectroscopy is fundamentally based on light scattering and acoustic wave interactions within a material, sample opacity limits penetration of the probing laser beam and produces internal scattering, which confounds Brillouin signals and measurement. A strategy to improve sample transparency for optical microscopy is known as optical clearing. This uses a combination of techniques to homogenize the refractive index of the overall sample. These include: delipidation, the removal of the lipids that contribute to opacity using solvents, urea or detergents; dehydration, hyperhydration or ‘refractive index matching’, whereby samples are soaked in high refractive index solutions that match the average refractive index of most biological tissues (between 1.44 and 1.52) [72]; or hydrogel embedding, where samples are incubated in a hydrogel and proteins are crosslinked prior to delipidation to preserve their structure [73].

Removal of structural components and equilibration of tissue with solvents and polymeric liquids can be expected to cause significant changes to mechanical properties of a sample. Riobibo et al investigated the effect of optical clearing of rat brain and heart tissues on Brillouin spectra and, surprisingly, observed only subtle differences between the BLS spectra of cleared and uncleared samples despite clear changes in the overall structure and morphology of the organs after clearing [74]. Furthermore, common tissue fixation with aldehydes is known to increase the stiffness of cell and tissue samples due to protein crosslinking [75–77]. As mechanical properties are intrinsically linked to material composition and structure, further studies are recommended to confirm the compatibility of sample clearing and preparation techniques with Brillouin microscopy and clarify the interpretation of results. It is also recommended that any sample preparation procedure that may affect the structural mechanics of samples in any way be thoroughly investigated and reported, including how samples are extracted, processed or mounted for Brillouin analysis. As an exemplar, Edginton et al provide a detailed protocol on how extracellular matrix (ECM) protein fibers were extracted and prepared for Brillouin measurement [78]. In the current status of the field, such methodology studies are necessary for meaningful comparison, evaluation of techniques and interpretation of results.
4. Progress in imaging cells, tissues and biological systems

Biological samples are inherently heterogeneous, structurally complex on a microscopic level and affected by temporal processes. It is increasingly recognized that mechanical properties of cells and tissues are integral and indicative of their function. However, as previously introduced, there has been a lack of appropriate means to measure mechanical information on a microscopic level across three dimensions. Brillouin spectroscopy combined with confocal microscopy has propelled the application of BLS for probing the viscoelastic properties of biological systems across a range of complexities, from single cell and subcellular components to whole tissues (figure 5 schematic), with the aim to become an established technique for better understanding structure-function relationships, fundamental cell biology, disease pathology and the growing field of mechanobiology. In [5], Prevedel et al discussed considerations for the application of BM for elucidating cellular responses to the mechanical properties of their immediate environments as well as for meaningful interpretation of Brillouin scattering signals. In this section, we summarize and discuss key developments in the application of BI for measuring the viscoelastic properties of biological samples and systems. The following section is presented in terms of both hierarchical complexity of samples studied to date, from ground matrix and supportive ECM to cells, tissues and organs, as well as historical progress based on technological capability.

4.1. Cellular and tissue components

Biological tissues are composed of organized groups of structurally and functionally similar cells held together by ECM. While ECM composition varies between tissues, the three major constituents are: (i) insoluble collagen fibers which provide structural strength, (ii) viscous proteoglycan proteins which cushion cells, and (iii) soluble adhesion proteins which bind proteoglycans and collagen fibers to cell receptors. The mechanical properties of the ECM of biological tissues are dependent on their constituent biopolymers and important for normal tissue function, where disturbances in these properties are key biomarkers of disease [32, 79]. Furthermore, the composition and organization of ECM components gives rise to unique structural-functional dependent mechanical properties of each tissue and therefore represents a key biological structure of interest in the study of tissue biomechanics.

Collagen is the most abundant structural protein of the ECM and collagen fibrils are the smallest load-bearing structural elements of tissues. Studying the mechanical properties of collagen in isolation can enable better approximation and modeling of the properties of the source tissue(s) of interest particularly if the composition of collagen is known or can be mapped. Indeed, the first application of BLS for biological analysis was described by Harley et al in 1977 in an attempt to correlate the molecular structure of rat tail collagen fibers to elastic moduli derived from Brillouin spectral measurements [80]. In this work, the elastic moduli of tropocollagen molecules in dry collagen were calculated using other known metrics such as density, peptide molecular weight and an estimate of hydrogen bond force constants. Harley et al then examined the effect of hydration on measurements of dry collagen and reported hysteresis and lower measured modulus with hydration of the collagen fibrils as well as an inverse relationship between the degree of hydration and sound velocity. The study also showed that the high-frequency micro-Young's modulus of collagen fibers is an order of magnitude greater than that obtained from the Hooke’s law region of a stress-strain curve, suggesting frequency-dependent molecular-scale viscoelastic properties. Cusack et al extended these findings to include measurements of the anisotropy of collagen fibrils by measuring the velocity of propagation of longitudinal and transverse polarized elastic waves at different angles to the fiber axis. These were combined to determine the elastic constant of collagen [81].

Similarly, Randall et al sought to determine the effect of fiber orientation with BFS in wetted rat tail collagen and horse hair keratin [22]. Differences in peaks with scattering vectors parallel, perpendicular and at a 45° angle with the length of the fiber were demonstrated, with implications in fiber structure and probing direction on Brillouin measurements. Lees et al similarly measured BLS across wetted collagen and various dried and/or mineralized tissues including antler (bone),ibia and tendon and compared sound velocity resolved into axial and radial components for each material [82]. Optical refractive index of mineralized samples was found via Brillouin scattering. While mineralization is not standard practice for tissue sample preparation for biological characterization, the technique enabled sample compatibility with the optical systems of that time.

More recently, Palombo et al characterized dehydrated collagen and elastin, major constituent proteins of ECM, using micro-focused BLS and reported Young's moduli values of approximately 10 and 6 GPa for collagen and elastin respectively [53] which are in line with previous findings. Advances in combinative systems have enabled more detailed correlative analysis. In [61], Mercatelli et al describe application of BLS, Raman spectroscopy and second-harmonic generated maps in combination to examine sutural lamellae,
collagenous structures within the cornea, in order to determine structure-function relationships in healthy and diseased states.

In addition to ECM mechanics, the presence and properties of pathological proteins are implicated in certain diseases and ageing processes. Antonacci et al studied the biomechanical properties of stress granules containing FUS proteins, implicated in amyotrophic lateral sclerosis (ALS), within whole cells using a unique background-deflection Brillouin (BDB) system [83] and found an increase in stiffness and viscosity of stress granules containing ALS mutated FUS proteins.

It is expected that collateral advances in hardware capability, experimental methodology and signal analysis will enable finer discretization and resolution of proteins and ECM components within living cells and tissues. In [69], Bevilacqua et al achieved sub-micron resolution in application of BLS to map changes in the mechanical properties of ECM in live zebrafish during development on sub-micron resolution, highlighting the importance of selecting optimal imaging parameters and spectral analysis for successful application of Brillouin microscopy (BM) as well as demonstrating the potential of BLS as a valuable tool for elucidating the role of ECM biomechanics in developmental and mechanobiology.

### 4.2. Biological systems

Here we focus on Brillouin analysis of *in vitro* models, which encompasses mammalian and bacterial cell cultures across an increasing level of complexity from single cell to multicellular models, as well as relevant tissue-analogue systems.

#### 4.2.1. Single cells

The cell is the fundamental unit of all living organisms. The ability of modern cell culture techniques to sustain cells *ex vivo* and grow them in a controlled manner has underpinned a large part of modern medical research. Brillouin analysis of single cells is currently still at an early stage however. At the time of this review, reports in the literature have primarily been for proof-of-concept and spatial resolution testing during system development. The primary aim has been to demonstrate successful resolution of subcellular structures with ever improved clarity, which is essential for enabling detailed biomechanical studies of cells on submicron levels. It is expected that much can be yet discovered from a single cell to organ level using the continuously improving variations of BM.

In one of the earliest applications of BM to single cell analysis, Scarcelli et al sought to determine the sensitivity of BM to osmotic factors, which affect the water content and organization of cytoplasmic structures within cells and therefore measured stiffness [84]. In [6], subjecting NIH 3T3 cells to hyperosmotic shock by increasing sucrose concentration to culture media was shown to induce a significant increase in stiffness measured across individual cells immediately after sucrose treatment, with a linear increase in stiffness with increasing sucrose concentration; these findings implicate consideration of the ionic and osmotic conditions of culture in experimental design and interpretation of BM measurements.

The nucleus is the largest and most rigid structure within eukaryotic cells, and therefore its physical properties contribute critically to the overall properties and mechanical behavior of cells. The structure and mechanical properties of the cell nucleus are known to regulate gene expression and key cellular processes such as proliferation, migration and differentiation [85]. Nuclear mechanics vary throughout the cell cycle and are important indicators of healthy and diseased states [86], but are very challenging to measure directly using physical means such as AFM. The non-contact nature of BM has positioned it as an advantageous tool for detailed study of nuclear mechanics. With sufficient resolution, BLS can be an effective contrast mechanism for differentiating nucleus from cytoplasm. In [7], Antonacci et al successfully resolved nucleoli from the nuclear membrane from cytoplasm of porcine aortic cells. The work also measured a 3.6% decrease in cytoplasmic stiffness (to 2.51 ± 0.03 GPa) in cells exposed to latrunculin-A, a drug that lowers cell stiffness by preventing cytoskeletal assembly, verifying the suitability of BM as a non-contact and non-destructive technique for measuring nuclear and intracellular mechanics.

Combined or correlative systems extend simple Brillouin analysis by providing an additional metric for mapping properties across cells. Mattana et al also differentiated nucleus from cytoplasm, in this case using a Brillouin-Raman system which correlated viscoelastic properties with biochemical composition. A significant reduction in cell stiffness was measured following transfection and oncogene expression [36], supporting the technique as an early biomarker for cancer detection. As mentioned earlier, Elsayad et al have engineered an integrated fluorescence emission-Brillouin imaging configuration along with its namesake technique (FBI), which is shown to enable simultaneous visualization and mechanical mapping of structures within cells at submicron resolution [62].

The ability to measure changes in the stiffness across cells in a contact-less, label-free manner opens up a plethora of Brillouin analysis-based applications for study of cellular biomechanics in disease and therapeutic development. Zhang et al demonstrate the extension of Brillouin microscopy to a flow cytometry technique.
capable of classifying cell populations based on nuclear mechanical signatures when flowed through a microfluidic device [87]. In [8], treatment of fibroblast cells with trichostatin-A, a chromatin decondensation drug, was shown to cause a significant reduction in nuclear stiffness, allowing successful differentiation from populations of untreated cells. It is expected that as BM becomes established as a technique for studying the biomechanics of cells and tissues, more applications will emerge, e.g. complementing single-cell omics.

4.2.2. Spheroids and organoids
Beyond a single cell, cell spheroids represent a more realistic in vitro tissue model compared to 2D culture and are currently the gold standard for therapeutic screening. Spheroids are clusters of cells induced to grow in a spherical formation by physical manipulation techniques, e.g. round bottom microwell plates, hanging droplet culture and encapsulation [88–91]. These cell aggregates represent micro-tissues and their 3D nature presents challenges for mechanical characterization and optical imaging throughout the entirety of a spheroid while simultaneously preserving its functionally relevant structure, presenting yet another opportunity for BI. Margueritat et al explored application of BLS for mapping across cancer spheroids and demonstrated a clear delineation between the looser peripheral layer of cells and stiffer core of a spheroid [11]. Similarly, Conrad et al measured the Brillouin frequency shift range of an ovarian cancer model cultured within a 3D gel matrix (Matrigel) during spheroid formation in a standard low-adhesion round-bottom plate as well as investigated the effects of hyposmotic and carboplatin treatment [68]. Increasing osmolality was shown to increase Brillouin frequency shift measured from the cancer nodule cultures, corroborating previous findings [6], while carboplatin, a chemotherapeutic agent, was shown to decrease Brillouin frequency shift in treated groups due to disruption of the structural integrity of the nodules. It is expected that scanning capability coupled with improved signal deconvolution and 3D volume rendering will enable full realization of BI for detailed study of spheroids and organoid in vitro models as well as their responses to therapeutic agents in drug screening.

4.2.3. Tissue analogue models
The field of tissue engineering aims to recapitulate functional biofidelic tissues and organs using a combination of biomaterials, cells and relevant factors. Also known as tissue constructs, tissue analogues and tissue phantoms, engineered tissues currently serve as a more ethical and readily producible platform for drug screening, disease modeling, or for patient-specific organ replacement. While many methods to fabricate 3D engineered tissues exist, bioprinting is the most recent development in enabling the generation of complex structures. While a variety of biomaterials are compatible with bioprinting technologies, hydrogels have been the most commonly investigated due to their biocompatibility and proximity of their physical characteristics with ECM. These hydrogels, also known as ‘bio-inks’, are composed of natural, synthetic or composite biomaterials that may be chemically or crosslinked via conjugation with a photo-initiator to form controlled structures. Being soft and viscoelastic, hydrogel mechanics are difficult to characterize conventionally through rheology which only allows assessment of bulk volumes. Given that cells are sensitive to local environmental cues, there exists an immediate opportunity to utilize BM for studying and characterizing properties of bioprinted hydrogel constructs on a microstructural level that is directly relevant to the cells. This has the potential to provide more conclusive insights into cellular responses and construct remodeling processes. In [92], Correa et al demonstrated an efficient Brillouin data collection and image analysis workflow on collagen gelatin hydrogels with different stiffness created by varying crosslinker concentration and formalin concentration. This is valuable for analysis of biologically-relevant tissue analogues with physiological hydration levels and subjected to routine fixation methods.

4.2.4. Biofilms
Biofilms are an organized collective of single-cell microorganisms of the same or different types that cohesively coexist within a shared self-excreted ECM sheet or membrane, wherein cells are attached to each other and frequently on surfaces. Microorganisms that typically form biofilms include bacteria, fungi and protists; a common example of a biofilm in the body is dental plaque. Biofilms also form on medical implants, can pose significant health risk, and are difficult to remove. Understanding the mechanical properties of biofilms will enable development of treatments that disrupt the ECM and stability of the biofilm, preventing its formation or facilitating removal from implant surfaces. Karampatzakis et al used BM to measure the internal stiffness of live P. aeruginosa biofilms under continuous flow and found that stiffness tended to increase towards the center of smaller colonies (films), indicating higher structural complexity akin to a spheroid in 2D [70]. Extension of BM with endoscopic techniques may enable real-time in situ study of biofilm formation on medical implants.
4.3. Tissues and organs
The initial success of BLS-based single cell studies sparked rapid progression to Brillouin imaging of tissues and more complex biological systems. Tissues are organized groups of similar cells that together, perform specific functions. There are four main types of tissues within the body: epithelial, connective, muscular and neural, whereby the composition, organization and orientation of cells defines each tissue and their unique functions. Organs are comprised of two or more tissues that operate in an anatomically distinct unit. Here we summarize Brillouin studies of biological samples within the structural complexity range of tissues to whole organisms.

4.3.1. Cornea and eye
The cornea is the clear frontal component of the eye which acts as a protective cover for the iris, pupil, lens and anterior chamber of the eye. The cornea is composed of water, collagens and glycan proteins. Likewise, the lens is also composed of collagens and glycosaminoglycans, with a uniquely crystalline structure that focuses light onto the retina as the basis of the mechanism of sight. Being transparent and therefore possessing high optical clarity as requisite for optical imaging, the cornea and lens are among the first tissues measured with Brillouin imaging systems. Randall et al were among the first to report Brillouin spectral measurements of the cornea and lens of the human eye along with those of a variety of vertebrate species [23, 24].

Scarcelli and Yun described the first in situ Brillouin measurement of the lens within a mouse eye [3] and stiffness map of ex vivo cornea [51] to set the precedent for subsequent studies of ocular tissue mechanics using Brillouin microscopy. Distinct differences in biomechanical properties between normal corneas and keratoconus were measured via a line-scanning Brillouin setup [9]. Given the high water content of the cornea, Shao et al determined the sensitivity of BM in detecting changes in hydration level within corneal tissues to facilitate analysis and interpretation of results [46]. In [93], Scarcelli et al applied BI to assess the efficacy of corneal stiffening via collagen crosslinking, a therapeutic means to halt the progression of keratoconus and corneal ectasia, on porcine cornea. Together, these results support the establishment of a number of clinical trials to evaluate and validate BI as a tool for diagnosing ocular anomalies [12]. In [13], Shao et al describe the first clinical trial and protocol using BI to map across corneas in vivo, where increasing biomechanical inhomogeneity was found to occur within the cornea with progression of keratoconus, as well as asymmetry in mechanical properties between the left and right cornea at the onset of the condition. This study was then extended, where Seiler et al measured a significantly lower Brillouin frequency shift in the thinnest regions of keratoconus corneas compared to normal corneas (5.7072 ± 0.0214 vs 5.7236 ± 0.0146 GHz, P < .001) [67].

While promising, clinical BLS measurements of corneal or ocular tissue necessitate consideration and resolution of two important issues: (i) the intrinsic risk of laser irradiation damage to the eye and (ii) motion-induced vibrational noise. More advanced optical technologies able to provide improved signal to noise ratio will enable a safe operational laser power compatible with live measurements of ocular tissues. In addition, future improvements to signal detectors and the development of more sophisticated processing algorithms will be able compensate and filter out natural movements and vibrations. These considerations will be further discussed in section 6.

4.3.2. Bone and cartilage
Bone and cartilage are specialized forms of connective tissue that provide structural support for muscles and organs within the human body. Bone is hard, highly vascularized and calcified connective tissue that comprises the skeleton whereas cartilage is soft, flexible, avascular, tissue that serves to absorb shock in the joints between bones, or forms rigid, low-load bearing structures such as within the nose, ear and larynx. The structure and mechanical properties of bone and cartilage are integral to their function, and disruption of these properties is implicated in injury and degenerative conditions such as arthritis and osteoporosis. Earlier detection of changes in the structure and strength of bone and cartilage will facilitate earlier diagnosis, which is key to effective treatment and improved patient outcomes.

The function-dependent structure of bone trabeculae present challenges to measurement of mechanical properties. Extending preliminary work in applying BLS to measure the viscoelastic properties of bone and cartilage [94], Matsukawa et al performed a comparative study with scanning acoustic microscopy (SAM) to determine the sensitivity of BLS in detecting anisotropy and decalcification, hallmarks of bone diseases [66]. Bovine trabecular bone samples were prepared in thin 30–150 μm slices for transparency, with some treated with ethylenediamine-tetraacetic acid (EDTA) to model decalcification. Brillouin spectra were site-matched with SAM; a significant reduction in frequency shift (expressed as wave velocity) following decalcification was measured as well as higher signal intensity due to optical clearing effects of demineralization, demonstrating the applicability of BLS for characterizing bone quality in disease and healing processes.
Bone remodeling phenomena, particularly in the presence of implants, remain poorly understood due to the lack of means of evaluating implant stability and osseointegration in a non-invasive manner during healing post-implantation. To address this, Matthieu et al investigated the capability of BLS in differentiating between mature and newly formed bone during post-implant healing based on viscoelastic properties and histological analysis [95]. Results indicated lower mineralization in newly formed bone compared to mature cortical bone, along with higher heterogeneity which reflects the initial lack of organization of ECM proteins during healing. This work confirmed the sensitivity of BLS in detecting mechanically-relevant compositional changes in bone during remodeling.

Akilbekova et al similarly employed BM to investigate the load-dependency and viscoelastic properties of bone during healing. In [96], the efficacy of bone morphogenetic proteins (BMPs) and stem cell-loaded heparin-conjugated fibrin (HCF) hydrogels in the repair of critical bone defects in a rabbit model was assessed by measuring changes in BFS and linewidth, which are indicative of the rate of bone formation and healing processes at the site of the defects. Results were correlated to x-ray images, where Brillouin frequency shift and linewidth increased with formation of an organized endosteal callous and showed that BMPs coupled with stem cells facilitated bone regeneration which is in line with previous findings.

Wu et al also applied Brillouin microscopy to detect the loss of ECM proteins in cartilage as occurs in the pathogenesis of osteoarthritis. In [14], ex vivo porcine articular cartilage subjected to trypsin digestion for 4 h yielded a 150 MHz decrease in BFS compared to untreated controls, confirming break down of structural ECM proteins; this was attributed to a small 4% increase in water content following enzymatic treatment. Overall, these studies support the potential of BI as a non-invasive tool for characterizing mechanical properties of bone and cartilage during formation and healing, as well as a method for early detection of changes in these properties in disease.

4.3.3. Epithelium
Epithelium is the most abundant of tissues, which covers the body and forms the inner and outer lining of the majority of organs and internal cavities. Epithelial tissues are characteristically comprised of tightly packed cells on a basement membrane and perform a variety of functions including physical protection, secretion, absorption, excretion, filtration, diffusion, and sensory reception. Disruption of the mechanical integrity and physical properties of epithelia is implicated in a number of diseases. Palombo et al performed the first correlative Brillouin, Raman and FITR analysis of ex vivo Barrett’s esophageal tissue [60, 65] and demonstrated the capability of such a system for simultaneous site-matching of mechanical and chemical signature maps, which is valuable for detecting compositional and structural changes in esophageal epithelia from chronic acid reflux as occurs in Barrett’s esophagus and other conditions such as cancer.

4.3.4. Cancers
Abnormal cellular functions and the uncontrolled division of these abnormal cells underpin a group of diseases referred to as cancer. Changes in gene expression, morphology and function in cancerous cells lead to distinct changes in the composition (biochemical, structural) and mechanical properties of tissues. Associated pathological changes in tissue stiffness would provide a diagnostic tool for early detection of cancerous and pre-cancerous cells and represent a potential application for Brillouin imaging. Troyanova-Wood et al applied BM to study ex vivo samples of malignant melanoma in a porcine model and measured a distinct change in BFS between melanomas (8.55 ± 0.18 GHz) and healthy skin tissues (7.97 ± 0.02 GHz), with regressing tumors between this range (8.11 ± 0.07 GHz) [64], demonstrating the capability of BLS for differentiating between melanoma and normal skin, with potential for more rapid and non-invasive diagnosis of melanoma and other skin cancers.

While tissue stiffness is known to be important for cancer progression, metastasis and patient outcome [97, 98], there is increasing evidence that the mechanical interaction between cells and ECM may also influence cancer development and progression [99, 100]. However, these micromechanical properties and interactions are difficult to analyze with current technologies. Therefore, in addition to diagnostic applications, BI can be implemented to better understand the interplay between cell-matrix interactions in cancer development and progression for the development of more targeted and effective therapies.

4.3.5. Plaques
Disease processes can produce aggregates of matter known as plaques and are characteristic of respective pathological conditions; examples include dental plaque, amyloid proteins in Alzheimer’s disease, psoriatic skin lesions and atherosclerotic plaque. As plaques accumulate during disease progression, they tend to become semi-hardened over time and disrupt the elasticity and functionality of the tissue in situ. Mattana et al reported the first application of BM in combination with Raman spectroscopy to map the viscoelastic profiles of individual amyloid beta protein plaques in ex vivo histological sections of mouse hippocampus.

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tissue [54] and showed, through spectral deconvolution algorithms, that amyloid plaques are comprised of a rigid core of β-folded (β-amyloid) proteins in a sheet conformation surrounded by a softer ring-shaped region richer in lipids and other protein conformations [101]. A known limitation with all optical methods is the penetration depth within a tissue, restricting their application to in vivo monitoring of plaque formation. However, BLS can still be a valuable means of understanding the biomechanics of plaque formation in amyloidopathy in biopsy samples as well as in evaluating the efficacy of treatments.

Atherosclerotic plaque is the hallmark presentation of atherosclerosis, where the endothelial wall of blood vessels is disrupted, allowing infiltration of lipoproteins that progressively accumulate and form a necrotic core [102]. Given that rupture of an atherosclerotic lesion leads to acute cardiac events and frequently death, early detection and treatment of rupture-prone plaques is key to patient survival. Meng et al [103] and Antonacci et al [15] investigated BM for giving quantitative measures of stiffness across rupture-prone atherosclerotic plaques, which are characterized by a thin, stiff fibrous cap covering a soft lipid-rich necrotic core within an arterial wall. They measured distinct differences in the mean frequency shifts of ex vivo plaque (15.79 ± 0.09 GHz) compared with normal vessel sections (16.24 ± 0.15 GHz) and showed a direct correlation between lower frequency shift for lipid-rich regions and higher for collagenous areas of the fibrous cap [15]. This study supports the combination of Brillouin spectroscopy with intravascular imaging to improve detection of vulnerable plaques and predict rupture potential in atherosclerotic patients.

4.3.6. Embryonic development
Mechanical forces have profound effects on cell behavior in adult tissues, but less is known about their roles during embryonic development where an embryo undergoes very dynamic changes in cell number, cell shape, cell contacts and migration to form functional organs and tissue structures [104]. Introduction of mechanical stimuli or chemical agents can disrupt highly sensitive processes within an embryo and therefore confound measurement, calling for a non-contact and label-free means of studying the biomechanical properties of cells and developing tissues during embryogenesis. Raghuhanthan et al demonstrated the efficacy of Brillouin microscopy in combination with optical coherence tomography (OCT) to map and measure changes in stiffness of organs within a developing embryo [63], where OCT provides structural guidance for correlating Brillouin measurements. Using this technique, structures such as the neural fold and developing heart and closed neural tube could be successfully identified. Zhang et al extended this work to investigate neural tube formation during embryonic development [71], further validating that Brillouin-OCT provides sufficient sensitivity and resolution to measure changes in stiffness of tissues and structures and its application in embryonic mechanobiology.

5. Data acquisition, processing and analysis

5.1. Data acquisition and processing
Data acquisition, storage and processing in BLS experiments is typically done by custom-built scripts and software, developed by each research group to meet particular requirements [92] with the exception of GHOST (developed by Table stable Ltd) and THAT ec software packages designed to automate alignment and data acquisition of TFP interferometers.

Curve fitting is one of the most important signal processing routines necessary to obtain the Brillouin frequency shift Ω and the FWHM of the peaks Γ. Several models can be used to achieve this goal, with Lorentzian, Damped Harmonic Oscillator (DHO) and Gaussian models being the most commonly applied. The TFP interferometer and nonlinear coherent techniques are able of achieving high spectral resolution of approximately 1–50 MHz whereas typical spectral features from a sample are a few hundred MHz in width. Therefore, the latest generation of instruments can be used to resolve ‘true’ line shapes unobstructed by the instrument function [2].

The hyperspectral analysis to decompose overlaid spectral features of Brillouin peaks has recently been applied to study amyloid-beta plaques in Alzheimer’s disease brains [101]. This technique, previously found to be useful to extract information in Raman imaging, has strong potential in Brillouin imaging of heterogeneous environments within the scattering volume such as majority of biological tissues and cells.

Another significant development in Brillouin data processing area is the spectral phasor analysis method in which the signal is represented as a combination of an amplitude and a phase [105]. This technique can be particularly useful in noisy measurements for improving signal contrast and for high-speed acquisition scenarios since it does not require the use of least square numerical fitting algorithms.

5.2. Models to interpret Brillouin scattering data in biological matter
Historically, the physics of BLS was first applied to understanding photon-phonon interactions and structural relaxations in gases, liquids and solid state crystalline materials, in which the stress-strain...
relationship can be described by the models of an ideal gas, a Newtonian fluid or a linearly elastic solid, respectively [106]. Recent application of BLS to study biomechanics of cells, tissues, matrix-like biomaterials and whole organisms, has challenged physicists and life scientists, calling for new theories that can explain BLS data and connect findings with other available methods of micromechanical testing such as AFM micro-indentation, optical tweezers, and micro-rheology. The search for new hybrid models that can explain BLS measurements in biological matter is one of the greatest challenges in the field, and the solution to this problem is much needed in order for the technology to be recognized by the rest of scientific community and translated to commercial applications. There have been a number of proposals that attempted to interpret BLS data by adopting viscoelastic or poro-elastic models and we briefly review these in the next sections.

### 5.2.1. Viscoelastic model

Viscoelasticity is the property of materials that exhibit both elastic and viscous characteristics when undergoing deformation. This dual nature can be described by setting elastic moduli to be complex and frequency-dependent. The complex longitudinal modulus $M' (\omega) = M'(\omega) + j M''(\omega)$ is then composed of the storage $M'(\omega)$ and the loss $M''(\omega)$ components where $\omega$ is the driving frequency of mechanical deformation. The former describes elastic material response to deformation, and the latter refers to energy dissipation due to viscous effects. According to this model, Brillouin peaks can be reproduced by a damped harmonic oscillator (DHO) function

$$I(\omega) = \frac{I_0}{\pi} \frac{\Gamma \Omega^2}{(\omega^2 - \Omega^2)^2 + (\Gamma \omega)^2}$$

(4)

convolved with the measurement instrument function. The Brillouin frequency shift $\Omega$ and line width $\Gamma$ derived from this model yield the storage and loss moduli at the peak frequency $\omega = \Omega$

$$M'(\Omega) = \frac{\rho}{q} \Omega^2 = \rho \nu^2,$$

(5a)

$$M''(\Omega) = \frac{\rho}{q} \Omega \Gamma = \eta \Omega,$$

(5b)

where $\eta = \frac{\rho}{q} \Gamma$ is the longitudinal kinematic viscosity. Importantly, $\Omega$ and $\Gamma$ are not independent parameters but depend on the wavelength of the probing laser $\lambda$ (equation (2)) and the ambient temperature $T$.

The single peak analysis shown above is the standard data analysis method in BLS applied to biology and biomedical fields. Nevertheless, there are a few other functions adopted for fitting Brillouin data, namely Lorentzian and Gaussian [2], and the choice of the fit model should be based on specific details of the experiment.

The frequency dependence of the storage and loss moduli in equation (4) and (5) is an important consideration. BLS experiments probe acoustic phonons in GHz frequency range and hence the measured frequency shift $\Omega$ and line width $\Gamma$ correspond to the material response at supersonic frequencies. Frequency dispersion of viscoelasticity manifests itself in drastically different response to stress perturbations below and above the structural relaxation frequency $\omega_r$, which is unique for each material. An intuitive explanation for material response below and above the relaxation frequency $\omega_r$ was presented by Palombo and Fioretto in their recent review [2].

It is worth noting that the supersonic frequency regime relevant to BLS experiments typically corresponds to material response above $\omega_r$. Under GHz perturbation, the material does not have enough time to relax and appears stiffer (higher values of $M'$) compared to the perturbation of the same amplitude but much lower frequency (Hz-kHz), typical of micro-rheology or micro-indentation measurements. Because the two groups of methods, low and high frequency, assess the relaxation dynamics of a system in two limits, these methods should be viewed as complementary to each other. It has been shown, however, that changes in storage and loss moduli resulting from pathological processes or drug treatment do correlate across the broad frequency range, thus showing similar trends at Hz and GHz frequencies [6, 11]. However, this is merely a rule-of-thumb and correlations should be verified for each specific material platform and experimental conditions.

### 5.2.2. Poro-elastic model

Recently a new interpretation of BLS results in tumor spheroids has been proposed in which the poro-elastic nature of some biomaterials (e.g. hydrogels) and tissues is considered [11]. A poro-elastic material can be represented by an elastic porous frame immersed in a viscous fluid. The stress tensor $\sigma_{ij}$ and mean fluid
pressure \( p \) are estimated based on a minimal Darcy-scale poro-elastic model as a function of the strain tensor \( \varepsilon_{ij} \) and fluid content \( \xi \), written in index notation as

\[
\sigma_{ij} = K_u \varepsilon_{kk} \delta_{ij} + 2\mu \varepsilon_{ij} - \frac{K_u - K_d}{\beta} \xi \delta_{ij},
\]

(6)

where \( K_u \) and \( K_d \) are the undrained and drained bulk moduli, respectively, \( \mu \) is the shear modulus of the drained material and \( \delta_{ij} \) is the Kronecker delta.

The fluid is typically allowed to drain through open pores of the solid frame under application of stress. Depending on the stress rate and critical time required for fluid to drain out, different scenarios can occur. The long term stress-strain response of tissues simulates the drained case where the fluid is allowed to flow freely \( (p = 0) \) and gives access to \( K_d \). At the high-frequency limit, however, the fluid has no time to drain and becomes trapped in the pores \( (\xi = 0) \). A critical frequency \( \omega_c = 2\pi f_c \) differentiates between these two limiting regimes. It has been found that for hydrogels this frequency is expected to be in the range \( f_c = 1 - 200 \text{ GHz} \) [11]. This suggests that the photon–phonon interactions underpinning BLS can probe poro-elastic system dynamics close to the threshold between drained and undrained regimes.

It has been suggested that when approaching \( f_c \), variations in the longitudinal modulus and hence the Brillouin frequency shift can be interpreted as a variation in a volume fraction of circulating water \( \phi \), so that \( M' \sim M'_w/\phi^3 \), with \( M'_w \) being the longitudinal modulus of fluid phase [11]. According to such interpretation, the increase in \( M' \) towards the center of the tumor spheroid could be related to the reduction in the amount of circulating water [11].

In general, the link between local hydration and the Brillouin frequency shift is well known in BLS community and has been studied earlier in relation to isolated collagen and elastin fibers [53], hydrogels [34] and cornea [46]. Both longitudinal \( M \) and Young’s \( E \) moduli are affected by fluctuations in tissue hydration and at GHz frequencies the effect of hydration becomes prominent for \( M \) in particular since fluid has no time to drain and behaves as solid.

### 5.2.3. Two-phase mixture model for highly hydrated biomaterials

The question of local hydration and its effect on the longitudinal and Young’s moduli has been explored for hydrogels with liquid contents above 90% [34]. Previously, the correlation between \( M \) and \( E \) was inferred based on a phenomenological formula

\[
\log M' = a \log E' + b
\]

(8)

obtained in a set of experiments using porcine and bovine lens tissues [45]. Equation (8) provided a simple relation to connect changes in \( E \) measured by conventional means of mechanical testing (unconfined compression test or AFM indentation) with changes in \( M \) measured by Brillouin microscopy. This, however, required verification for a broader range of materials and material parameters.

In [34], Wu et al. set to examine the universal validity of equation (8) in a set of experiments with hydrogels of two compositions, i.e. polyethylene oxide (PEO) and polyacrylamide (PA). Since swelling is a common feature of hydrogels (as well as tissues and cells), the gel hydration level \( \epsilon \) was measured and kept as an independent parameter. The hydration level in both types of gels was varied between 90% and 100%. Both \( M \) and \( E \) were influenced by \( \epsilon \), but more importantly no single law correlation was found between \( M \) and \( E \). For example, the same value of \( M \) was measured for PEO hydrogels with molecular weights of 1, 4 and 8 MDa but an independent measurement of \( E \) using an unconfined compression test indicated a five-fold difference in the Young’s modulus between gels of 1 and 8 MDa.

To explain the results of Wu et al. one can adopt the poro-elastic model with fluid content of \( \epsilon > 0.9 \). Due to high fluid content, equations (6) and (7) can be significantly simplified, leading to a biphasic model of hydrogels proposed in earlier works by Hosea [107] and Johnston [108]. According to that, the aggregate compressibility of the gel, \( \beta_{agg} = 1/M_{agg} \), is a superposition of individual compressibilities of its two phases, a fluid phase with compressibility \( 1/M_f \) and a solid one with compressibility \( 1/M_s \)

\[
\frac{1}{M_{agg}} = \frac{\epsilon}{M_f} + \frac{1 - \epsilon}{M_s},
\]

(9)

Note that \( M_s \) is the longitudinal modulus of the hydrated solid polymer network (that includes contribution of fluid bound to the network) rather than the longitudinal modulus of bulk dry polymer this
network is made of. The linear relation described by equation (9) is clearly an approximation and does not take into account many important factors of hydrogel viscoelasticity, e.g. any interaction between fluid and solid fractions, the internal geometric structure of the pores, pores size, the number of crosslinks or the network topography. The biphasic model should only be applied in the limit $\epsilon \rightarrow 1$, in which it proves to be quite useful in the explanation of Brillouin scattering data from highly-hydrated materials such as hydrogels [25] and cornea [30].

6. Challenges for clinical application of Brillouin Imaging

Brillouin imaging is rapidly becoming useful as a research tool for studying structure-function relationships in biological cells and tissues. Although in a nascent stage, the field is already characterized by a great diversity of approaches and custom equipment. While increased accessibility to optical components has given rise to rapid development of new techniques and system configurations, such multiplicity raises a particular risk. Namely, the scientific relevance of any Brillouin light scattering-based study is as good (or lacking) as the alignment between (1) the research question, (2) system capabilities, (3) the measurement protocol, (4) the assumptions made about the studied sample, and (5) the models and algorithms used to analyze the raw Brillouin spectral data. Furthermore, Brillouin imaging in itself is not without its challenges, which are presented categorically in the following sections.

6.1. Sample preparation methods

Biological tissues have complex microarchitectures and are not optically clear. Clearing reagents and protocols have been proposed, however, clearing treatments and even conventional tissue fixation methods can be expected to disrupt and alter the physical properties of cells and tissue components. The effects of the sample preparations procedures need to be investigated in the context of BLS and imaging and caution should be taken in the interpretation and extension of results measured from fixed and treated ex vivo samples to be the same as in vivo tissue properties. While it is currently possible to image live specimens whose thickness or optical clarity permit sufficient optical penetration e.g. embryos during early stages of development [71] or small and transparent zebrafish [69], the majority of BI studies are performed on fixed or ex vivo samples due to the issue of optical clarity. Therefore, the possible impact of sample preparation, handling and storage techniques on subsequent mechanical properties must be carefully established. Live in vivo or in vitro imaging, or other techniques that negate the need for chemical or physical modification of a sample will allow measurement of more physiologically accurate information.

6.2. Analysis and interpretation of Brillouin data

Elucidation of Brillouin data sampled across a focal volume can become non-trivial in heterogeneous tissue samples. Nevertheless, it is possible to correlate Brillouin frequency shifts with different components within a heterogeneous sample using multispectral reconstruction numerical techniques and additional imaging modalities, e.g. Brillouin–Raman imaging. Furthermore, it is likely that signal deconvolution algorithms will be continually refined and optimized to enable conclusive, site-matched measurements.

While Brillouin frequency shift has increasingly been used interchangeably with ‘stiffness’ by biologists, other material parameters are required in order to calculate longitudinal modulus, an accepted mechanical metric, from Brillouin frequency shift. These include refractive index and density, which are challenging to measure for composite and heterogeneous materials such as biological matter or even an individual cell. While several methods for measuring refractive index exist, e.g. digital refractometers, there must be a means of conveniently measuring refractive index in a site-matched manner across 3D samples to enable accurate approximation of longitudinal moduli with sufficient spatial resolution. One way of overcoming this is to directly measure refractive index within a Brillouin system. Fiore et al recently devised a means to simultaneously measure refractive index using a dual co-localized BLS configuration [109].

As the Brillouin community grows, there could be a collaborative effort to develop an open-source library of accepted values for different tissue types and components to simplify modeling and extraction of longitudinal moduli from Brillouin spectra. Otherwise, Brillouin frequency shift and linewidth in themselves could potentially become accepted standalone unitized metrics with implicit/direct correlation to longitudinal modulus wherever relative (not absolute) measures of stiffness suffice e.g. for assessing changes in cell or tissue stiffness post-treatment.

6.3. Standardized protocols for BLS measurements

At present, the design of Brillouin systems is in a developmental stage with the aim to optimize and refine particular aspects of BI as a technique, e.g. resolution, or exploratory in developing further techniques e.g. combinative systems. However, as hardware and system configuration determines capability and results,
there exists a need for a comparative study to identify system variability in order to enable meaningful comparison of results obtained from a diverse range of system designs; this could be done by say, measuring a set of standard samples (distilled water, ethanol, selected cell lines) across different instruments as a first step towards standardization.

Once instrument variability is addressed, the consensus on standardized protocols for measuring different categories of biological samples of interest will facilitate the community to achieve reproducible measurements and allow for more ready comparison of data between researchers. These protocols can be further developed and refined for BI measurements of tissues in order to first determine reliable statistical thresholds of ‘normal’ and ‘abnormal’ Brillouin results for each tissue type, using parameters that can be clearly differentiated at the 95% confidence level (or better), before extension to disease detection and clinical diagnosis. Finally, a clear guide for selecting Brillouin components, e.g. laser wavelength, laser power and spectrometer type to satisfy majority of biological and biomedical experiments could be useful for non-experts in the field.

7. Conclusion

Owing to its inherent advantage of non-contact measurement, Brillouin imaging offers the capability to provide unique insights into the physical properties of biological cells and tissues. However, use of Brillouin imaging in a diagnostic capacity is still in its infancy with several issues requiring attention. Firstly, the instrumentation itself will need to become more standardized, economical and smaller as well as redesigned with clinicians as the end-user, i.e. more ergonomic with user-friendly data acquisition and handling interfaces. The issue of mechanical robustness of any machine that includes a Fabry–Perot etalon needs to be considered due to the latter containing relatively delicate moving components. Protocols for sample preparation and measurement will need to be standardized and consolidated across a range of instrument designs. Reliable statistical envelopes for the BFS maps of ‘normal’ or ‘abnormal’ tissues will need to be determined for selected medically important pathologies through larger-scale clinical trials in order to develop diagnostic algorithms which can then be packaged as software to complement commercialized clinical systems.

Although we have shown in this review that BLS-based imaging and spectroscopy can offer useful data for a great range of medical studies, it will only be feasible to commercialize the techniques for a few conditions at first, and then likely only in the form of ex vivo biopsies for non-surface/deep tissues. Tumor detection and growth, characterization of systemic sclerosis, or monitoring of plaque formation, as examples, may offer suitable targets for the initial transfer of this technology to clinical settings. The ultimate challenge, however, is to combine this technique with endoscopy for routine in vivo measurements in clinical settings. Given the valuable insights that Brillouin imaging can produce and how rapidly the field has progressed in recent years, we have little doubt that this goal will be reached in time.

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Statement of Contribution

CP devised the direction and theme of the review, designed the main structure and outline of the document, wrote sections 3.3, 4 and 6, helped write sections 1, 3.2 and 7 and provided general editing. JC edited the document, provided general editing and feedback on section 4. MC edited the document, provided critical insights and some additional references, and helped write sections 6 and 7. IK wrote sections 1–3 and 5, helped write section 6 and 7, provided general editing and contributed with critical planning and discussion of the manuscript.

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