High-sensitivity and high-specificity biomechanical imaging by stimulated Brillouin scattering microscopy

Itay Remer1,2✉, Roni Shaashoua1, Netta Shemesh2, Anat Ben-Zvi3 and Alberto Bilenca4,14✉

Label-free, non-contact imaging with mechanical contrast and optical sectioning is a substantial challenge in microscopy. Spontaneous Brillouin scattering microscopy meets this challenge, but encounters a trade-off between acquisition speed and the specificity for biomechanical constituents with overlapping Brillouin bands. Stimulated Brillouin scattering microscopy overcomes this trade-off and enables the cross-sectional imaging of live Caenorhabditis elegans at the organ and subcellular levels, with both elasticity and viscosity contrasts at high specificity and with practical recording times.

Label-free biomechanical imaging approaches include a variety of techniques, such as atomic-force microscopy and optical coherence elastography1,4, that probe biomechanics at high spatial resolution in complementary regimes, but require application of an external force on the sample. Second-harmonic generation microscopy5 circumvents this necessity, yet its applicability is limited to a small number of structural proteins. An emerging technique, spontaneous Brillouin scattering microscopy6–15, enables label-free and non-contact biomechanical imaging in three dimensions by measuring the so-called Brillouin shift (ΩB) and linewidth (ΓB), which correspond to the frequency shift and linewidth of light that is backscattered inelastically from gigahertz-frequency longitudinal acoustic phonons that are characteristic of the mechanical components of the material. Although spontaneous Brillouin microscopes exhibit a large free spectral range, they often sacrifice acquisition speed for mechanical specificity, or vice versa, depending on whether a scanning spectrometer (slower but with higher spectral resolution) or a non-scanning spectrometer (lower spectral resolution but faster) is being used6–15.

Here, we introduce stimulated Brillouin scattering (SBS) as a process that simultaneously enables practical acquisition times and high mechanical specificity in Brillouin microscopy of biological samples. SBS is a photon–phonon scattering process that is analogous to stimulated Raman scattering (SRS) but involves lower-frequency acoustic phonons16. In frequency-domain SBS, counter continuous-wave (CW) pump and probe beams at frequencies ωp and ωs overlap in the sample to efficiently interact with a longitudinal acoustic phonon of frequency Ωp (Fig. 1a). When ωp is scanned around the Stokes frequency (ωs − Ωp), the probe intensity (I) at ωs experiences a stimulated Brillouin gain (ΔI, SBS) via wave resonance, where the pump intensity Ip at ωp shows a stimulated Brillouin loss (SBL; Fig. 1b). The opposite occurs when ωp is scanned around the anti-Stokes frequency (ωs + Ωp). No gain or loss arises at ωp otherwise. Thus, unlike spontaneous Brillouin scattering, SBS and SBL enable spectral measurements that are free of elastic background without sacrificing acquisition speed for mechanical specificity, or vice versa6–15.

There is a close relationship between the SBS and the SBL spectra and the high-frequency complex longitudinal modulus M* of the sampling volume, which reads as ρ × (λp / 2n)2 × Ωp2 × (1 + (Γp / Ωp)), where ρ is the probe wavelength, and n and ρ are the refractive index and the mass density of the medium17. The different physical nature of M* and the conventional low-frequency viscoelastic moduli leads to a large difference in their values, and does not allow for a direct interpretation of M* in terms of the Young’s modulus18. Yet, empirical correlations have been found between M* and these low-frequency mechanical moduli1. As in two-photon fluorescence and SRS, the volume sampled in SBS is diffraction limited, as SBS and SBL depend on the product of the pump and probe intensities focused at the sample.

Although frequency-domain and impulsive SBS were used for Brillouin imaging of tissue phantoms at high spectral resolution, they exhibited limited detection sensitivity and/or spatial resolution, hindering imaging of biological systems20–22. To enable biomechanical SBS imaging, we constructed a SBS microscope (Fig. 1c and Supplementary Fig. 1) based on our previous SBS spectrometer design16, but with improved spatial resolution (~0.8 × 0.8 × 16 μm3, organ level; ~0.3 × 0.3 × 2 μm3, subcellular level) and shot-noise sensitivity. This enhanced sensitivity led to spectrum-acquisition times that were as low as 20 ms in the semitransparent C. elegans model organism and 2 ms in water (Supplementary Note 1 and Supplementary Fig. 2), using a total near-infrared excitation power of ~265 mW at the sample, and without obvious photodamage in C. elegans (Supplementary Note 2 and Supplementary Fig. 3). With scanning and non-scanning spontaneous Brillouin microscopes, spectrum-acquisition times as low as 10 s or 20–50 ms, respectively, can be used in microorganisms or cells and tissues, with ~10- to 60-mW visible excitation powers23–27. The effective spectral resolution of the SBS microscope was below ~39 MHz at the organ level, and below ~98 MHz at the subcellular level (Supplementary Note 3), whereas those of scanning and non-scanning spontaneous Brillouin microscopes are ~100 MHz and ~700 MHz, respectively28–31. Taken together, these results demonstrate that SBS overcomes the trade-off between the spectrum-acquisition time and the spectral resolution (mechanical specificity) in spontaneous Brillouin scattering.

We acquired SBS spectra measured from the pharyngeal region of a live wild-type C. elegans young adult (Fig. 1d and Supplementary

1Biomedical Engineering Department, Ben-Gurion University of the Negev, Be’er-Sheva, Israel. 2Agilent Research Laboratories, Petach Tikva, Israel. 3Department of Life Sciences, Ben-Gurion University of the Negev, Be’er-Sheva, Israel. 4Ilse Katz Institute for Nanoscale Science and Technology, Ben-Gurion University of the Negev, Be’er-Sheva, Israel. ✉e-mail: remeri@post.bgu.ac.il; bilenca@bgu.ac.il
We validated the effectiveness of SBS microscopy for biomechanical imaging using cross-sectional measurements of the high-frequency viscoelastic response to hyperosmotic stress of the pharynx and the surrounding tissue in live wild-type *C. elegans* young adults (Fig. 1f,g). Such measurements are important because *C. elegans* naturally live in the soil and are known to experience body stiffening under hyperosmotic stress. The SBS data show that $\Omega_B$ of the pharyngeal region rose with increasing osmolarity because $\Omega_B$ correlates with higher stiffness in hydrated biological systems, our data indicate that hyperosmotic conditions increased pharyngeal stiffness. We observed a similar trend for the pharyngeal viscosity, with larger $\Gamma_B$ at higher osmolality (Fig. 1f,g).

Fig. 1 | Principle and method of SBS microscopy. a, Acousto-optic interaction between focused, counter-propagating pump ($\omega_1$) and probe ($\omega_2 < \omega_1$) light with a longitudinal acoustic phonon ($\Omega_B$) via optical interference (stripe pattern) and acoustic resonance (gray wavefronts). b, Intensity transfer between the pump ($I_1$) and the probe ($I_2$) light by virtue of SBS along an interaction length $l$, with an intensity increase in the probe beam ($\Delta I_2$, SBG) and an intensity decrease in the pump beam (SBL). Frequency (left) and spatial (right) domain illustrations are shown. c, In the SBS microscope, the pump and probe beams are focused to the same point in the sample (S). Backscattered SBG is detected on the probe intensity by a custom transimpedance photoreceiver (TIR) and a lock-in amplifier (LIA). Also measured is the sample attenuation. By scanning $\omega_2$ around $\omega_1 - \Omega_B$, a SBG spectrum is acquired. OM, optical modulator; L, lens; OF, optical filter; $\lambda/4$, quarter-wave plate; PC, personal computer. d, SBG spectrum $G(\Omega)$ of the pharynx (top) and the surrounding tissue (bottom) of a *C. elegans* nematode ($n=10$) from locations marked by red and blue crosses in the coregistered brightfield image (T, measurements in dots and fit in a black line; N, nematode fitted component in magenta; B, buffer fitted component in cyan). e, Three-dimensional sections of $\Omega_B$, $\Gamma_B$, and $G_B$ through the nematode’s head in d. f, Representative $\Omega_B$, $\Gamma_B$, and $G_B$ images ($n=10$) along with coregistered brightfield images of the head of live *C. elegans* young adults under isotonic (left) and hypertonic (middle and right) salt conditions. g, Mean values of $\Delta I_2$ and $\Gamma_B$ calculated across the pharynx (P, purple) and the surrounding tissue (S, cyan) of the nematodes at different osmolarities ($n=10$ nematodes per osmolarity value, $P$ value between osmolarity levels < 0.001, using one-way analysis of variance following post hoc Tukey’s test; $P$ value within osmolarity levels < 0.0005, using paired two-tailed Student’s t-test). Error bars are s.d. of the mean. All data were acquired from the middle plane of the nematodes (25-µm depth) with acquisition times of 20 ms per 4-GHz spectrum at organ-level resolution, and were fitted by double Lorentzian. All image sizes are 100 × 200 pixels, with scale bars of 20 µm.
Next, we used SBS microscopy to quantify the high-frequency complex longitudinal modulus of live wild-type *C. elegans* at the L2 stage. We obtained images of $\Omega_i$, $\Gamma_i$ and $G_0$ along with the coregistered brightfield image of a live *C. elegans* larva (Fig. 2a–c). Using the triplet $\Omega_0$, $\Gamma_0$ and $G_0$ and the relation between mass density and SBG\(^{16}\) with the reported refractive index distribution of *C. elegans*\(^{14}\), we evaluated the spatial distributions of the complex longitudinal moduli of the nematode (Fig. 2d,e). We computed the mean values of these moduli and of $\Omega_i$ and $\Gamma_i$ across the volumes of the pharynx and the surrounding tissue in several nematodes (Fig. 2f,g).

Taken together, these results suggest that the larva pharynx was stiffer (larger $M'$ and $\Omega_i$) and more viscous (larger $M''$ and $\Gamma_i$) than the surrounding tissue, which is likely attributable to the muscular nature of the pharynx. Therefore, SBS microscopy has the capacity to map the high-frequency complex longitudinal modulus of live organisms, opening possibilities for the study of biomechanics at high frequencies.

Finally, we performed large-area biomechanical imaging of developing *C. elegans* with SBS microscopy. We mapped in vivo the distributions of $\Omega_i$ and $\Gamma_i$ across the middle plane of nematodes...
at the L2, L3, L4, young adult and adult stages (Fig. 2h–j). These maps show organs and structures, such as the pharynx, gonad and eggs, within the nematodes based on the viscoelastic contrast. The mean values of $\Gamma_B$ and $\Gamma_\Omega$ of the pharynx and the surrounding tissue computed from these maps (Supplementary Fig. 6) indicate that the pharynx was stiffer and more viscous (larger $\Gamma_B$ and $\Gamma_\Omega$) than the surrounding tissue at all developmental stages. Moreover, the pharyngeal region maintained a relatively constant viscoelasticity during development, suggesting that its mechanical properties are determined early in development, perhaps owing to the prominent function of the pharynx in feeding. We also observed the development of the reproductive system of C. elegans (Fig. 2h–j). To visualize this system with subcellular mechanical specificity, we reduced the sampling volume using lenses with high numerical-aperture values (Supplementary Notes 1–4). We obtained $\Omega$ and $\Gamma_\Omega$ maps across the gonad arm of an adult nematode (insets in Fig. 2i,j). We also recorded similar maps from sequential depths of the gonad (Supplementary Fig. 7). These maps revealed the nuclei and the nucleoli of germ cells and growing oocytes in the gonad. Each nucleus was surrounded by a cytoplasm, where the nucleoplasm was softer and less viscous (smaller $\Omega$ and $\Gamma_\Omega$) than the nucleolus and the oocyte cytoplasm. We also observed an oocyte pushed from the spermatheca into the uterus, with increasing stiffness and viscosity (larger $\Omega$ and $\Gamma_\Omega$) at its center, likely due to the deformation of the oocyte through the small aperture of the spermatheca. Lastly, we identified a four-cell embryo in the uterus, with a nucleoplasm that was also softer and less viscous than the oocyte cytoplasm.

Whereas our SBS microscope operates in transmission mode, SBS in reflection mode may be possible in turbid medium, similar to measurements performed by SRS microscopy. Spontaneous Brillouin line-scanning microscopy could, where CW SBS would be faster than CW SRS yet slower than pulsed SRS. 

SBS in reflection mode may be possible in turbid medium, similar to measurements performed by SRS microscopy. Spontaneous Brillouin line-scanning microscopy could, where CW SBS would be faster than CW SRS yet slower than pulsed SRS. 



## Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41592-020-0882-0.

Received: 2 September 2019; Accepted: 27 May 2020; Published online: 3 August 2020

## References

1. Thomas, G., Burnham, N. A., Camesano, T. A. & Wen, Q. Measuring the mechanical properties of living cells using atomic force microscopy. *J. Vis. Exp.* **56**, 50497 (2013).
2. Kennedy, B. F., Wijesinghe, P. & Sampson, D. D. The emergence of optical elastography in biomedicine. *Nat. Photonics* **11**, 215–221 (2017).
3. Chen, X., Nadiarynhk, O., Plotnikov, S. & Campagnola, P. J. Second harmonic generation microscopy for quantitative analysis of collagen fibrillar structure. *Nat. Protoc.* **8**, 654–669 (2012).
4. Palombo, F., Madami, M., Stonesc, N. & Fioretto, D. Mechanical mapping with chemical specificity by confocal Brillouin and Raman microscopy. *Analyst* **139**, 729–733 (2014).
5. Scarcelli, G. et al. Noncontact three-dimensional mapping of intracellular hydromechanical properties by Brillouin microscopy. *Nature Methods* **12**, 1132–1134 (2015).
6. Antonacci, G. et al. Quantification of plaque stiffness by Brillouin microscopy in experimental thin cap fibroatheroma. *J. R. Soc. Interface* **12**, 20150483 (2015).
7. Elsayad, K. et al. Mapping the subcellular mechanical properties of live cells in tissues with fluorescence emission-Brillouin imaging. *Sci. Signal* **9**, rs5 (2016).
8. Scarponi, E. et al. High-performance versatile setup for simultaneous Brillouin-Raman microspectroscopy. *Phys. Rev. X* **7**, 031015 (2017).
9. Mattana, S. et al. Non-contact mechanical and chemical analysis of single living cells by microspectroscopic techniques. *Light Sci. Appl.* **7**, 17139 (2018).
10. Akikibekova, D. et al. Brillouin microscopy and radiography for assessment of viscoelastic and regenerative properties of mammalian bones. *J. Biomed. Opt.* **23**, 1–11 (2018).
11. Schlüßer, R. et al. Mechanical mapping of spinal cord growth and repair in living zebrafish larvae by Brillouin imaging. *Biophys. J.* **115**, 911–923 (2018).
12. Andriotis, O. G. et al. Hydration and nanomechanical changes in collagen fibrils bearing advanced glycation end-products. *Biomed. Opt. Express* **10**, 1841–1855 (2019).
13. Nikolić, M. & Scarcelli, G. Long-term Brillouin imaging of live cells with reduced absorption-mediated damage at 660 nm wavelength. *Biomed. Opt. Express* **10**, 1567–1580 (2019).
14. Zhang, J. et al. Tissue biomechanics during cranial neural tube closure measured by Brillouin microscopy and optical coherence tomography. *Birth Defects Res. B* **111**, 991–998 (2019).
15. Prevedel, R., Diz-Muñoz, A., Ruocco, G. & Antonacci, G. Brillouin microscopy: an emerging tool for mechanobiology. *Nat. Methods* **16**, 969–977 (2019).
16. Boyd, R. W. *Nonlinear Optics* (Academic Press, 2008).
17. Remer, I. & Bilenca, A. Background-free Brillouin spectroscopy in scattering media at 780 nm via stimulated Brillouin scattering. *Opt. Lett.* **41**, 926–929 (2016).
18. Remer, I. & Bilenca, A. High-speed stimulated Brillouin scattering spectroscopy at 780 nm. *APL Photonics* **1**, 061301 (2016).
19. Vaughan, J. M. & Randall, J. T. Brillouin scattering, density and elastic properties of the lens and cornea of the eye. *Nature* **284**, 489–491 (1980).
20. Ballmann, C. W. et al. Stimulated Brillouin scattering microscopic imaging. *Opt. Exp.* **25**, 18139 (2017).
21. Ballmann, C. W., Meng, Z., Traverso, A. J., Scully, M. O. & Yakovlev, V. V. Impulsive Brillouin microscopy. *Optica* **4**, 124–128 (2017).
22. Krug, B., Koulourakis, N. & Czarnecki, J. W. Impulsive stimulated Brillouin microscopy for non-contact, fast mechanical investigations of hydrogels. *Opt. Exp. Express* **27**, 26910–26923 (2019).
23. Park, S. I., Goodman, M. B. & Pruitt, B. L. Analysis of nematode mechanics by piezoresistive displacement clam. *Proc. Natl Acad. Sci. USA* **104**, 17576–17581 (2007).
24. Choi, W. et al. Tomographic phase microscopy. *Nature Methods* **4**, 717–719 (2007).
25. Freudiger, C. W. et al. Label-free biomedical imaging with high sensitivity by stimulated Raman scattering microscopy. *Science* **322**, 1857–1861 (2008).
26. Hu, C. R. et al. Stimulated Raman scattering imaging by continuous-wave laser excitation. *Opt. Lett.* **38**, 1479–1481 (2013).
27. Zhang, J., Fiore, A., Yun, S. H., Kim, H. & Scarcelli, G. Line-scanning Brillouin microscopy for rapid non-invasive mechanical imaging. *Sci. Rep.* **6**, 35398 (2016).

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
Methods

SBS microscope. A detailed schematic of the SBS microscope is shown in Supplementary Fig. 1a. An amplified CW distributed-feedback (DFB) laser (SYT TA-pro-DFB, Topica) and a CW DFB laser (SYT DE-100-DFB, Topica), both s-polarized, thermally stabilized and coupled into a polarization-maintaining single-mode fiber, serve as a pump beam at frequency \( \omega_1 \) and a probe beam at frequency \( \omega_2 \), respectively. The frequency \( \omega_0 \) corresponds to the wavelength of 780.24 nm, and the frequency \( \omega_0 \) is scanned around the Stokes frequency \( \omega_0 - \Omega_\text{B} \) (with \( \Omega_\text{B} \) being a characteristic longitudinal Brillouin shift of the material) to acquire a SBG or SBL spectrum. The SBG or SBL spectrum is given by \( g(\Omega) = \Delta I/ I_1 = g \times g(\Omega) \times I_1 \times I_2 \), where \( \Delta I \) is the SBG or SBL experienced by the intensity of the probe beam \( I_2 \), \( g \) is the crossing efficiency of the pump and probe beams in the sample, \( g(\Omega) \) is the SBG gain (plus sign) or loss (minus sign) factor well represented by a Lorentzian line shape, \( I_1 \) is the interaction length of the two counter laser beams in the sampling volume and \( I_2 \) is the intensity of the pump beam. Although \( \Delta I \) and \( \eta \) provide equivalent SBG spectra because the passband of the detection filter used is flatter for the Stokes than for the anti-Stokes frequency bands. The lasers exhibit mode-hop-free tuning ranges larger than 50 GHz and linewidths smaller than ~2 MHz, providing wide spectral range and high spectrometer resolution for the acquisition of SBG spectra.

The frequency of the probe laser is scanned by modulating its current with a sawtooth wave from a function generator (AFG2021, Tektronix). The frequency difference between the pump and probe lasers, \( \Delta \omega = |\omega_0 - \omega_\text{B}| \), is determined from a lookup table produced by measuring the beat frequency of the two lasers as a function of the probe laser current with a fast photodetector (1434, Newport) and a frequency counter (Model 524C, Stanford Research Systems). Phase-sensitive detection of the probe beam, filtered by a narrowband Bragg filter (SFC-780, Optigrate), suppresses the amplified spontaneous emission of the pump laser. The filtered beam is modulated with an acousto-optic modulator (12510, Gooch and Housego) driven by a 1.1-MHz sinusoidal wave from the function generator. The same sinusoidal wave is used as a reference for the lock-in amplifier. Unlike the low-frequency lock-in detection scheme previously used in SBS microscopy, we implement lock-in detection at megahertz frequencies for improving the sensitivity of SBS, as we have used in our previous SBS spectrometer\(^{11,12}\). Following modulation, the pump beam is p-polarized by a half-wave plate, and is transmitted toward the sample through a polarizing beam splitter. The pump and probe beams are circularly polarized orthogonally to each other with quarter-wave plates, and are tightly focused at a joint point in the sample. This polarization scheme enables an increase in the cross filtering efficiency of the pump and probe beams in the sample and isolates the pump and probe optical paths from each other. Aspheric lenses with a numerical aperture (NA) of 0.25 (Aspheric) are used for imaging at the organ level, and objective lenses with 0.7 NA (Olympus) are used for subcellular imaging.

As a non-linear process, SBS yields \( 2 \Delta \omega / (4 \pi \text{NA}) = 0.78 \mu m \) at an estimated axial resolution of \( 1 \times n \times N_\text{A} = 16.6 \mu m \) in water. The crossing angle between the pump and probe beams is set to 180°, improving the crossing efficiency of the current \( \text{SBG} / 2 \) is collected with the same lens that focuses pump light into the path of the probe beam, and a complementary metal-oxide-semiconductor camera (L225, Lumenera) was imaging the sample through a dichroic mirror located immediately before the detection unit of the microscope, as shown in Supplementary Fig. 1a.

Preparation of the C. elegans samples. Wild-type (N2) C. elegans nematodes were grown on nematode growth medium (NGM) plates seeded with Escherichia coli OP50 or OP50-1 strains at 15 °C; 30–60 embryos, laid at 15 °C, were picked, transferred to new plates and grown at 25 °C for the duration of the experiment. We determined the developmental stage of the nematodes using a light microscope during the developmental time window of interest. During the reproductive period, animals were transferred to fresh plates every 1–2 d to circumvent progeny contamination.

For imaging at 0.25 (or 0.7) NA, we first prepared two 0.5-mm-thick (0.25-mm-thick) agar pads (5%) mixed with 10 mM sodium azide solution to anesthetize the nematodes. The agar pads were mounted on two 0.17-mm-thick round glass coverslips (18 mm and 25 mm in diameter), and 15–10 nematodes at a specific developmental stage were sandwiched between the agar-padded coverslips (Supplementary Fig. 4a). Ten microliters of an M9 contact buffer were added between the agar pads and served as a control environment for the nematodes. To fix the entire sample and to avoid dehydration, a few drops of ultraviole were applied at the edge of the smaller coverslip, and a thin layer of Vaseline sealed the gap between the two coverslips, as illustrated in Supplementary Fig. 4b. Following SBS imaging, nematodes were washed from the agar pads to NGM plates for recovery.

Estimate of the mass density and the complex longitudinal modulus of materials by SBS. In SBS, the mean absolute density of a material \( \rho \) is related to \( \Omega_\text{B} \) and \( \Gamma_\mu \) and the normalized line-center gain factor \( g_0 \) of the SBG spectrum of the medium by the expression

\[
\rho = \frac{\gamma 2 \omega_0 \Omega_\text{B} \Gamma_\mu}{2 \pi c^2 \Omega^2 + 4 \gamma^2 \Omega_\text{B}^2 g_0^2}
\]

where \( \gamma \) is the electrostrictive constant, \( \omega_0 \) is the frequency of the probe beam, \( \rho \) is the refractive index of the medium, \( c \) is the speed of light in vacuum and \( g_0 \) is the acoustic wavenumber. \( g_0 \) is calculated from the SBG spectrum \( g(\Omega) \) as

\[
g_0 = \frac{g_{\text{eq}}}{\Delta I_\text{eq}}
\]

with \( \Delta I_\text{eq} \) being the peak gain \( \text{SBG} / 2 \) and the crossing efficiency of the pump and probe beams in the sample, \( I_1 \) the intensity of the pump beam at the sample entrance, \( I_2 \) the equivalent SBS interaction length in the medium calculated on the
basis of the assumed spatial distribution of the pump intensity in the sample. For backward SBS, $q_b = 2\times \omega_x \times n / c,$ and equation (1) reduces to the Boyd version given as:

$$\rho = \frac{2\nu_i^2 \omega_i^2}{c^2 \Delta k_i} \Omega \left(1 + \frac{\Gamma_B}{\Omega_c^2}ight).$$  \hspace{1cm} (3)$$

By estimating $\gamma$ using the Lorentz–Lorenz relation, we obtained $\gamma = (n^2 - 1) \times \nu_i$ (ref. 15). Thus, the mean density of a material $\rho$ can be evaluated from the measured values of $\Omega_c$, $I_B$, and $G_0$ of the SBS spectrum of the material and from knowledge about the material refractive index $n$. This methodology offers a more robust approach for evaluating $\rho$ compared with other methods that estimate $\rho$ on the basis of the Lorentz–Lorenz relation and its similar forms, because they require, in addition to information about $n$, additional knowledge, for example, about the molecular weight, polarizability and refraction increment of the material. Given the spectral parameters $\Omega_c$ and $I_B$ of the measured SBS spectrum, $\rho$ estimated by equation (3) and the literature refractive index value of the material $n$, the complex longitudinal modulus of the material $M'$ is calculated as:

$$M' = \rho \frac{\nu_i^2}{\Omega_c^2} \left(1 + \frac{\Gamma_B}{\Omega_c^2}\right).$$  \hspace{1cm} (4)$$

with $\nu_i = \sqrt{n^2 - 1}$ and $\lambda_i$ denoting the wavelength of the beam at $\omega_0$ (ref. 13). We validated equation (3) and computed $M'$ (equation (4)) in various materials using the SBS microscope with 0.033-NA focusing lenses (Supplementary Table 2). The use of these very-low-NA lenses minimized the NA-induced spectral effects on the acquired SBS spectrum, enabling the fundamental validation of the accuracy and precision of our approach for the evaluation of the material density, and hence of the complex longitudinal modulus of the material. Literature refractive index values at a wavelength of 780.24 nm and at 20°C were used in equations (3) and (4) for the materials measured16,17. The estimated mass density of all materials agreed well, to within 1–10% with values from the literature (Supplementary Table 2). For water and agar, the mass densities were underestimated, possibly because the Lorentz–Lorenz relation (and hence the estimation of $\gamma$) is only an approximation for liquids and solids.

Estimate of the mass density and the complex longitudinal modulus in *C. elegans* by the SBS microscope. In backward SBS, the SBS spectrum of the nth voxel of volume $\Delta x \times \Delta y \times \Delta z$ centered at $(x_i, y_i, z)$ in a scattering medium is:

$$G(\Omega) = n g(\Omega) f_0 \left|I_0^{eq}\right|^2,$$  \hspace{1cm} (5)$$

where $g(\Omega)$ is the SBS gain factor which, to good approximation, is described by a Lorentzian with $\Omega_c$ and $G_0$ where $I_0^{eq}$ is the effective SBS interaction length in the medium:

$$I_0^{eq} = \mu_i^{-1}(x_i, y_i) \times \left(1 - e^{-\mu_i(x_i, y_i) \Delta z}/2\right).$$  \hspace{1cm} (6)$$

with $\mu_i(x_i, y_i)$ being the mean total attenuation coefficient of the medium at $(x_i, y_i)$. $I_0^{eq}$ as a function that describes the dependence of the SBS interaction length on the spatial distribution of the pump intensity in the sample. $I_1^B$ is the pump intensity at the voxel entrance:

$$I_1^B = I_{SBS} e^{\mu_i(x_i, y_i) \Delta z}/2,$$  \hspace{1cm} (7)$$

where $I_{SBS}$ is the pump intensity at the entrance plane of the medium.

To estimate the mass density of the *C. elegans* nematode at the nth voxel, we used equation (3) with the reported refractive index $n$ value of the region to which the voxel belongs in the nematode, $\Omega_c$ and $I_1^B$ retrieved from the SBS spectrum $G(\Omega)$ measured at the nth voxel and the normalized Brillouin line-center gain factor $g_0^{eq}$ evaluated as:

$$g_0^{eq} = \frac{G(\Omega_0)}{\Omega_0 f_0 \left|I_0^{eq}\right|^2}.$$

Here, the mean total attenuation coefficient $\mu_i(x_i, y_i)$, appearing in $I_0^{eq}$ (equation (6)) and $I_1^B$ (equation (7)), was recovered by measuring the attenuation of the probe beam through the nematode at $(x_i, y_i)$ using the DC output signal of the photoreceiver together with the estimation of the thickness of the nematode at the same point from the brightfield images (assuming cylindrical symmetry of the nematode’s body). The voxel’s axial position $z$ in equations (3) and (7) was approximated as the extent of the resolution cell along the axial dimension, and the voxel's axial position $z_i$ in Equation 7 was obtained from the position readings of the motorized sample stage.

To compute the complex longitudinal modulus of the material $M'$ at the nth voxel of the *C. elegans* nematode, equation (3) is used with the spectral parameters $\Omega_c$ and $I_1^B$ of the SBS spectrum measured at the nth voxel, the mass of density $\rho$ estimated at the voxel and the literature refractive index $n$ value of the region to which the voxel belongs in the nematode.

We point out that equation (5) assumes focusing lenses of low NA (<0.1). With increasing NA, $I_1^B$ increases and $g_0^{eq}$ decreases at a similar rate because the total SBS power remains approximately constant for similar pump and probe crossing efficiencies and laser powers at the sample. Consequently, the accuracy in evaluating $\rho$ is determined by the accuracy of $\Omega_c$ (equation (3)). Using focusing lenses of NA ≤ 0.7, we verified experimentally that the decrease in $\Omega_c$ is <2% compared with $\Omega_c$ at NA = 0.1, yielding a <2% decrease in the accuracy of $\rho$. For the precision of $\rho$, the fractional uncertainties of $\Omega_c$, $I_1^B$, and $g_0^{eq}$ add in quadrature, resulting in values comparable to the fractional uncertainty of $I_1^B$ (<10%).

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

All imaging and spectral data that support the findings of this study and which are not available from public repositories owing to university constraints are available from the corresponding authors upon reasonable request and with the permission of Ben-Gurion University of the Negev.

**Code availability**

The SBS image reconstruction codes that support the findings of this study are available from the corresponding authors upon reasonable request and with the permission of Ben-Gurion University of the Negev under the BSD license. The code used to analyze the spectral data is provided under the BSD license as Supplementary Software along with representative sample data. The LabVIEW program for controlling the microscope, which is hardware-dependent, is available from the corresponding author upon request.

**References**

28. Remer, I., Cohen, L. & Bilenuka, A. High-speed continuous-wave stimulated Brillouin scattering spectrometer for material analysis. J. Vis. Exp. 127, e55527 (2017).

29. Polanskiay, M. N. Refractive Index Database (2008–2019); http://www.refractiveindex.info.

30. Podek, J., Procházková, O. & Medin, A. Studies on agaroses: 1. Specific refractive index increments in dimethyl sulfoxide and in water at various wavelengths and temperatures. Polymer 36, 4967–4970 (1995).

31. Weast, R. C. Handbook of Chemistry and Physics (CRC Press, 1987).

**Acknowledgements**

A.B. acknowledges the support of the Israel Science Foundation (grant no. 1173/17). I.R. acknowledges the support of the Azrieli Foundation.

**Author contributions**

I.R. and A.B. initiated and supervised the project; N.S and A.B.-Z. developed the SBS microscope and analyzed the data; I.R. and A.B. wrote the manuscript and all the authors contributed to the manuscript.

**Competing interests**

I.R. is an employee of Agilent Technologies. All other authors declare no competing financial interests.

**Additional information**

Supplementary information is available for this paper at https://doi.org/10.1038/s41592-020-0882-0.

Correspondence and requests for materials should be addressed to I.R. or A.B.

Peer review information Nina Vogt was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a

☑ Confirmed

☐ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement

☐ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

☐ The statistical test(s) used AND whether they are one- or two-sided

Only common tests should be described solely by name; describe more complex techniques in the Methods section.

☐ A description of all covariates tested

☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons

☐ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)

☐ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted

Give P values as exact values whenever suitable.

☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

☐ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

In house custom made stimulated Brillouin scattering microscope was used for spectral and image data generation and collection (SBS microscope note 1 in methods section and supplementary Fig. S1). The image acquisition process was controlled by a custom program (LabVIEW version 2014, National Instruments).

Data analysis

Spectral analysis of acquired Brillouin spectra was performed using custom MATLAB codes (R2018b version, The MathWorks Inc.) and executed under a Windows 10 operating system on a standard personal computer (codes are available in Supplementary Software). The statistical analysis was performed with standard MATLAB functions in the statistical toolbox.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The data that support the findings of this study, associated with the figures listed below, are available from the corresponding authors upon reasonable request and with the permission of Ben-Gurion University of the Negev. Representative data can be downloaded from the Supplementary information.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☑ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-list.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | The sample size was chosen to be sufficiently large to demonstrate the feasibility of the method in biological samples. For instrument characterization, a large sample size was chosen to obtain a Normal distribution. |
|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data were excluded from the analysis. |
| Replication | All replication were successful. The exact number of replicates for each experiments is given in the figure legends. |
| Randomization | Random biological samples at a defined developmental stage were picked from agar plates and imaged by the microscope in the experiments described in the manuscript. No Randomization was applied during data analysis. |
| Blinding | The analysis was fully automated with the same analysis parameters for all datasets being compared and presented in the manuscript. |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|-------------------------------|--------|
| n/a involved in the study | n/a Involved in the study |
| ☑ Antibodies | ☐ ChiP-seq |
| ☑ Eukaryotic cell lines | ☑ Flow cytometry |
| ☑ Palaeontology | ☐ MRI-based neuroimaging |
| ☐ Animals and other organisms | |
| ☐ Human research participants | |
| ☑ Clinical data | |

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | The study involve the following C. elegans nematodes strains:  
|-------------------|-----------------------------------------------------------------------------------------------------------------------------------|
|                   | 1. N2  
|                   | 2. CL2070: dvl5/70 [hsp-16.2::GFP + rol-6[su1006]]  
|                   | 3. DM8005: rasls5 [myo-3p::GFP::myo-3 + rol-6[su1006]]  |
| Wild animals | The study did not involve wild animals |
| Field-collected samples | The study did not involve samples collected from the field |
| Ethics oversight | No ethical approval or guidance was required |

Note that full information on the approval of the study protocol must also be provided in the manuscript.