nature research

Peer Review Information

Journal: Nature Methods
Manuscript Title: Rapid biomechanical imaging at low irradiation level via dual line-scanning Brillouin microscopy
Corresponding author name(s): Jitao Zhang, Giuliano Scarcelli

Reviewer Comments & Decisions:

Decision Letter, initial version:

16th May 2022

Dear Giuliano,

Your Brief Communication, "Rapid biomechanical imaging at low irradiation level via dual line-scanning Brillouin microscopy", has now been seen by 3 reviewers. As you will see from their comments below, although the reviewers find your work of considerable potential interest, they have raised a number of concerns. We are interested in the possibility of publishing your paper in Nature Methods, but would like to consider your response to these concerns before we reach a final decision on publication.

We therefore invite you to revise your manuscript to address these concerns. I strongly recommend that you discuss a revision plan with me before embarking on any experiments.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

When revising your paper:

* include a point-by-point response to the reviewers and to any editorial suggestions

* please underline/highlight any additions to the text or areas with other significant changes to facilitate review of the revised manuscript

* address the points listed described below to conform to our open science requirements

* ensure it complies with our general format requirements as set out in our guide to authors at www.nature.com/naturemethods

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Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further. We look forward to seeing the revised manuscript and thank you for the opportunity to consider your work.

Best regards,
Nina

Nina Vogt, PhD
Senior Editor
Nature Methods

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The work by Zhang et al. combines a 780 nm line illumination microscope with a VIPA spectrometer to acquire Brillouin scattering light. Brillouin imaging was obtained by scanning and rotating the sample through the line illumination. While the results show low photodamage and an equivalent acquisition time of 1 ms per pixel with spectral precision of <10 MHz, the image quality and the lack of significant spatial Brillouin data arise major concerns about the applicability of the method for Brillouin microscopy and imaging of biologically relevant environments. Unless these concerns are addressed, I

cannot support publication.

Major concerns:

1. Fig. 1c, 2a-c, and 2f: It seems that the SNR/spatial resolution/spectral resolution (broadening)/confocality of the method and/or the illumination line size and/or the selected samples limit the quality of the images substantially. In particular, spatial information is lacking in all the images. A comprehensive study of these parameters is important in order to understand the applicability of the method for microscopy and imaging in biological settings where (sub)cellular resolution is critical.

The main niche of multiplexing imaging techniques, such as SPIM (Science. 2004 Aug 13;305(5686):1007-9, Cell Tissue Res. 2013 Apr;352(1):161-77, Optica Vol. 3, Issue 4, pp. 452-457, 2016), is in high quality imaging of thick and large samples such as embryos of hundreds of μm to mm in size for which confocal microcopy is not well suited; however, the quality of the images demonstrated in this work is not satisfactory; Can LSBM be shown to achieve at least the image quality of state-of-the-art confocal Brillouin microscopes (CBM) but with a 1 ms pixel acquisition time (Biomed Opt Express. 2019 Apr 1; 10(4): 1567–1580, Biomed Opt Express. 2019 Feb 25;10(3):1420-1431, J Biomed Opt. 2017 Aug;22(8):1-6)?

2. Fig. 1d: The attempt to compare between CBM and LSBM is appreciated, yet some important details are missing: Was the comparison made using the same scattering geometry? similar SNR and Brillouin shift precision? What was the acquisition time per pixel used with the confocal setup? How does the difference in these parameters and the different wavelength used affect the fair comparison between CBM and LSBM? Also, it seems that the sample was not imaged simultaneously, thus multiple independent experiments should be performed to show statistical insignificance between the methods.

3. Fig. 1e: What is the origin of the lower frequency Brillouin shift? Was it confirmed statistically in multiple independent experiments as the difference in frequency is around 100 MHz? Could it be confirmed also pixelwise rather than using the statistics from the whole Brillouin image? While two frequency components are observable in the CBM spectrum, their existence is unclear by inspecting only the LSBM spectrum. Why? What are the implications of this latter point on the accuracy of the measured LSBM spectra and the image quality?

4. Fig. 1g,h: A comparison between CBM and LSBM for sample viability as a function of light dose is important. What is the maximal light intensity in CBM at 780 nm to enable sample viability similar to that of LSBM? What is the best acquisition time per pixel that can be achieved by CBM at this maximal light intensity compared with that of 1 ms? What is the image quality and Brillouin shift at this maximal light intensity and best acquisition time per pixel compared with that of LSBM at 1 ms?

5. Fig. 2a-c: Why do no spatial features appear in the hypoosmotic sample? Is it due to the domination of water in the sample? Or is it related to the factors mentioned in comment 1 that mask the spatial features?

6. Fig. 2d,e and 2g,I,j: While the average and std of the Brillouin shift are important measures in the analysis of spatiotemporal processes, the described analysis lacks the spatial dimension that is required for a comprehensive understanding of spatiotemporal processes in biological samples. Can spatiotemporal information be provided by LSBM at each pixel (in addition to the radial averaging of

the Brillouin shift that adds spatial information but only marginally)? or perhaps LSBM can be principally used to parallelize measurements of processes with dynamics slower than the exposure time of the camera (for single line imaging)?

7. Fig 2g,h: Besides being noncontact, what are the advantages of imaging by LSBM over AFM at some points given that the LSBM images are essentially spatially averaged to obtain useful quantities?

8. How was the acquisition time per pixel of 1 ms in the spheroids selected? It seems that both the spheroid and water samples were measured with the same acquisition time per pixel of 1 ms; Was the SNR and Brillouin shift precision obtained in the spheroids similar to those measured in water although spheroids represent a complex medium while water a simple fluid? A methodological way for choosing the appropriate acquisition time in biological samples is missing.

9. Supplementary Fig. 3: It seems that there is a dependence of the spectral precision/resolution on the spatial position in the sample. Study of this point and its implication on the accuracy and precision of the Brillouin spectrum measurements should be performed.

Minor comments:

1. What are the cellular components shown in Fig. 1c, 2a-c, and 2f? How many pixels were recorded for a single image? What is the maximum frame rate experimentally achieved by LSBM?

2. Volume rendering of the image data set in Fig. 1c would be essential to clearly visualize the sample in 3D.

3. It seems that the scale bar information for Fig. 2f is missing in the caption.

4. How were the Bragg and Fabry-Perot filters stabilized with respect to the laser line?

5. It seems that there is a typographical error in the x-label of Supplementary Fig. 3a,b and it should be "spectral axis".

6. A title should be added to the color bar in Supplementary Fig. 3a

7. For clarity and consistency with Supplementary Fig. 3c, the spectral axis of Supplementary Fig. 3a,b should be presented in GHz. Are the counts in the y-axis of these figures represent electrons or photons? having these counts in photon numbers would be clearer.

8. The sample size of the AFM measurements is significantly lower than that of the Brillouin measurements. What is the effect of this difference on the statistical analysis/interpretation of the data?

9. Was the stated optical resolution measured? If not, this should be indicated explicitly.

10. Was shot-noise operation confirmed experimentally in the spheroids?
11. What is the purpose of the green light and O5 in Fig. 1b?

12. For clarity, the spatial and spectral axes should be denoted on the camera plane in Fig. 1b.

13. What was the spectral resolution of the VIPA spectrometer?

Reviewer #2:
Remarks to the Author:
A Summary of Key Results: The Brillouin microscope reported here uses line scanning from opposite directions and orthogonal detection to increase image speed to 1 ms per pixel and reduce dose rates to 7.4% of what would be required for conventional confocal point scanning. It achieves this based on spatial resolution of 1.5 μmx1.5 μmx4.8 μm, whilst achieving spectral extinction >70 dB and spectral precision of <10 MHz. Could the authors also please specify the field of view of their microscope? And can they also ensure FOV and voxel number is included or able to be readily deduced in each experimental case, as well as the total acquisition time. (At bottom of p2 Supplementary an (axial?) FOV of 150-300 μm variable is mentioned.)

The key results in this work are to show that Brillouin microscopy is viable on live spheroids over a time lapse of five days, and to demonstrate short term changes can induced by osmotic shock can be imaged, every minute over 13 minutes.

B Originality and significance: Line scanning in an orthogonal detection geometry was proposed by this group and published in 2016 – Sci Reports: Ref 25. To this geometry is added a counterpropagating illuminating beam and an elegant explanation of how the droop in Brillouin frequency with depth in the sample can be compensated. How do the authors justify this work relative to their 2016 work, and how do they rebut the claim of incremental improvement? And can the authors please reference the state-of-the-art they claim to improve upon (lines 67/68).

Overall, it is indeed encouraging to see Brillouin microscopy continue to progress as a non-contact mechanobiological imaging technique – I am supportive of publication, once minor issues are addressed and subject to a convincing explanation of novelty relative to Sci Rep 2016.

C Data & methodology: Mapping the multiple voxels axially along the line of illumination onto the spectrometer is termed multiplexing, which is a term that is widely used in other spheres. In particular, as regards simultaneous counter-illumination, it becomes confusing as to whether both beams illuminate simultaneously, or how each beam are sequentially switched on. Can the authors make this very clear throughout please. Can the authors briefly outline (perhaps in Supplementary materials) the extent to which the multiplexed spectrometer performance is compromised (or not) by the multiplexing. Can the wavelength of the laser implicit in using a Rb cell be explicitly stated when introduced please.

Whilst the droop in Brillouin frequency versus depth is a very interesting feature, most Brillouin measurements are relative rather than absolute – can a discussion of this point be included please. For example, under time lapse imaging as shape changes then intrinsic shift will change independent of local biomechanics. As well, time lapse at a single point implies looking for changes not absolutes. In general, this rather critical point should be further elaborated.

D Appropriate use of statistics: No specific comments.

E Conclusions: robustness, validity, reliability: Overall, this manuscript provides a modest but sufficient level of validation of its key findings, and provides most but not all key experimental details as commented on above.

F Suggested improvements: experiments, data for possible revision: Mention is made of the use of a tuneable lens to further reduce the illumination intensity five-fold – obvioulsy doing this would be a worthwhile exercise, but I would not see this as mandatory here.

G References: Appropriate credit is given to previous work.

H Clarity and context: I found the abstract rather weak and did not convey the key novelty and achievements well at all.

Reviewer #3:
Remarks to the Author:
This paper from Zhang et. al. presents an improved multiplexed Brillouin microscopy technique based on line-scanning and single-shot spectral analysis. The authors expand a previously developed line-scanning setup (Zhang et. al., Scientific Reports 2016) by equipping it with a dual-line illumination that enables artifact-free imaging of thicker samples. Importantly, the new method greatly improves the speed of acquisition allowing fast 2D/3D imaging of biological samples with minimal photodamage. This is supported by several experiments demonstrating the capabilities of LSBM for mapping mechanical properties of living tissue: the authors demonstrate 3D Brillouin mapping of spheroids and characterize sample viability and proliferation following irradiation. Furthermore, they show that the method can be used to capture dynamic changes in tissue mechanical properties by imaging spheroids after a hyper/hypo-osmotic shock and comparing the evolution of mechanical properties in healthy and tumor spheroids over time.
Overall, the new method presented here by Zhang and colleagues overcomes current limitations of Brillouin microscopy that have hindered its wide application in mapping mechanical properties of living tissues and will be of great interest to a wide community. I therefore recommend publication once the points below are addressed:

Major points:
1. Since the speed of acquisition is one of the main strengths of the new method, it would be helpful to refer to the overall acquisition times more explicitly in the text. For example, it would be informative to compare the overall acquisition times of the images in Figure 1 panels c and d. This will allow readers to grasp more easily what spatio-temporal scales can be imaged with the presented method. In the same way, the choice of illumination time for the photodamage experiment should be justified (would 5 minutes be a typical acquisition time?).
2. The negative control used in Figure 1g should be specified in the text / Figure legend together with its N number.
3. The statement that authors are able for the first time to observe stiffening of growing tumor spheroids, guided by a stiff core is perhaps slightly overstated. It would be better to rephrase this part (lines 154-156), putting it in the context of several studies looking at mechanical properties of tumors organoids/spheroids that have previously highlighted the presence of a stiffer "core" (Han et al, Nature physics 2020, Mahajan et al, Cancers 2021).
4. In the last section, authors could further expand the discussion of strengths and limitations of the

technique: for example, lines 145-149 are quite vague ("analysis of different sample types", "different mounting configurations"). We would encourage the authors to be more explicit in this discussion as it will allow a broader readership to evaluate the applicability of the method presented to different systems and questions of interest (some interesting points could be a discussion of the size/thickness of samples, possibility to image developing tissues/embryos, combining the system with other imaging modalities like fluorescence).

Minor points:
1. Line 46, the references provided for examples of mechanically-relevant in vivo processes are somewhat narrow in scope, it would be useful to add references to a more extensive discussion of the topic (eg reviews)
2. We would encourage the authors to consider sharing the code developed for this project on an open-source platform such as GitHub, instead of making it available upon request. This is especially true for the image fusion code which could be helpful to other researchers in the community interested in adopting a dual-line illumination scheme.

Author Rebuttal to Initial comments

Response to comments

We thank all the reviewers for valuable comments. In the revision we have addressed all their concerns with new experimental data, new analysis and manuscript modifications. Thanks to these revisions, we believe the manuscript has significantly improved. All the changes in the manuscript and supplementary material are highlighted in red color. Below are the detailed point-by-point responses.

Reviewer #1:

Remarks to the Author:

The work by Zhang et al. combines a 780 nm line illumination microscope with a VIPA spectrometer to acquire Brillouin scattering light. Brillouin imaging was obtained by scanning and rotating the sample through the line illumination. While the results show low photodamage and an equivalent acquisition time of 1 ms per pixel with spectral precision of <10 MHz, the image quality and the lack of significant spatial Brillouin data arise major concerns about the applicability of the method for Brillouin microscopy and imaging of biologically relevant environments. Unless these concerns are addressed, I cannot support publication.

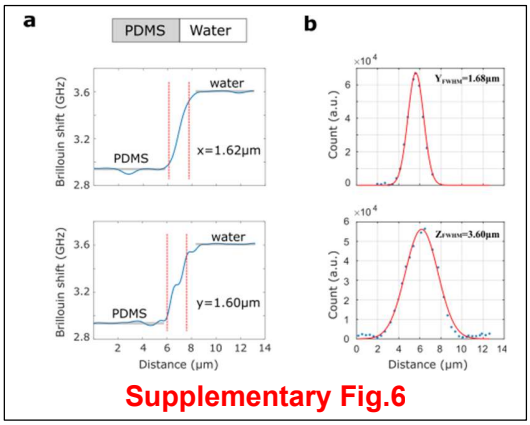
Response: We thank the Reviewer for providing a thorough analysis of our manuscript. We have addressed the Reviewer's concerns by collecting several more data sets and providing a quantitative and comprehensive characterization for phantoms and spheroids. We demonstrate that our dual linescanning Brillouin microscope (dLSBM) is diffraction-limited; the quality of a linescan Brillouin image

is equivalent to a confocal one of similar NA; and, it provides same spatially resolved information that is of high biological relevance. Below is a detailed response to Reviewer #1's comments.

Major concerns:

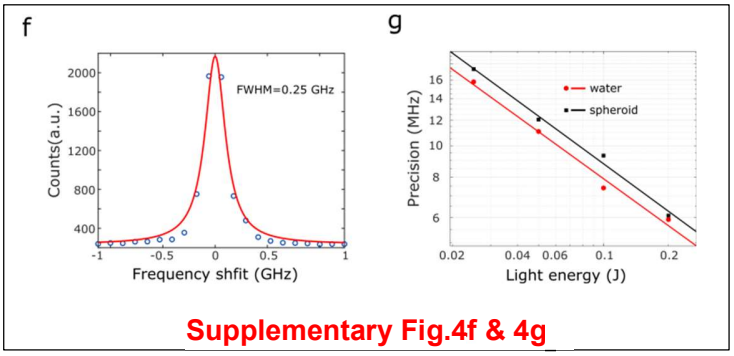
Comment 1: Fig. 1c, 2a-c, and 2f: It seems that the SNR/spatial resolution/spectral resolution (broadening)/confocality of the method and/or the illumination line size and/or the selected samples limit the quality of the images substantially. In particular, spatial information is lacking in all the images. A comprehensive study of these parameters is important in order to understand the applicability of the method for microscopy and imaging in biological settings where (sub)cellular resolution is critical. The main niche of multiplexing imaging techniques, such as SPIM (Science. 2004 Aug 13;305(5686):1007-9, Cell Tissue Res. 2013 Apr;352(1):161-77, Optica Vol. 3, Issue 4, pp. 452-457, 2016), is in high quality imaging of thick and large samples such as embryos of hundreds of µm to mm in size for which confocal microcopy is not well suited; however, the quality of the images demonstrated in this work is not satisfactory; Can LSBM be shown to achieve at least the image quality of state-of-theart confocal Brillouin microscopes (CBM) but with a 1 ms pixel acquisition time (Biomed Opt Express. 2019 Apr 1; 10(4): 1567–1580, Biomed Opt Express. 2019 Feb 25;10(3):1420-1431, J Biomed Opt. 2017 Aug;22(8):1-6)?

Response 1: The suggestion by Reviewer #1 to perform a comprehensive characterization of image parameters was well received. We have performed a thorough characterization of spatial resolution (**new Supplementary Fig.6**), spectral resolution (**new Supplementary Fig. 4f**), SNR and spectral precision in both test and spheroid samples (**new Supplementary Fig. 4e & 4g**). We quantitatively demonstrated that our dLSBM system is ***diffraction limited*** in terms of spatial resolution and ***shot-noise limited*** in terms of spectroscopy analysis; indeed, dLSBM provides spatial detail of information identical to an equivalent confocal system (CBM). Below are the set of critical new data supporting our claim:



Supplementary Fig.6

1A- To characterize the spatial resolution/confocality, we used two methods: (1) measuring the transition of two materials' interface; (2) measuring the Rayleigh scattering of a small bead (0.5 μm). The results show the lateral and axial resolutions are ~1.6 μm and ~4 μm, respectively (**new Supplementary Fig. 6** on the right). This is consistent with the collection NA (0.3) and the size of the illumination beam.



Supplementary Fig.4f & 4g

1B- To characterize the spectral resolution, we measured the linewidth of the Rayleigh scattering (so to avoid the convolution with the natural linewidth of Brillouin signatures), and the FWHM is 0.25 GHz. This is equivalent to the finesse of 40 of our VIPA etalon (FSR=10 GHz), which is consistent to the specification of the manufacturer, and comparable to state-of-the-art confocal instruments (**new Supplementary Fig. 4f**).

1C- We also characterized the SNR and spectral precision in both standard material (water) and spheroid (**new Supplementary Fig. 4g**). The results show our spectrometer is shot-noise limited in both test and spheroid samples, and 10 MHz precision can be achieved for spheroid experiment with acquisition speed of 1 ms/pixel.

Importantly, to demonstrate that dLSBM captured the spatially-resolved mechanics of biological samples, namely, **subcellular information equivalent to CBM** of same NA, we added the following evidence:

1D- Here we show a line plot of the representative Brillouin images of CBM and dLSBM (i.e., Fig.1d in main text) which clearly demonstrates how the mechanical contrast has micronscale spatial

dependence within spheroids (**Fig. 1.1D** on the right). The dashed line in the Brillouin image of the left panel indicates the location of the profile plot showing on the right panel. The line plot of the spatial mechanical change also indicates that dLSBM captured equivalent micron-scale information/resolution (e.g., soft-stiff transition line highlighted by the red background) to CBM with similar NA.

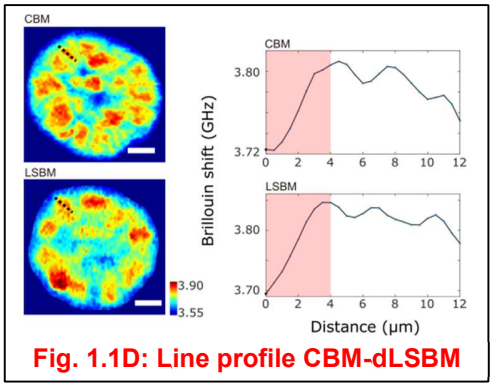


Fig. 1.1D: Line profile CBM-dLSBM

1E- We expanded (as Reviewer #1 explicitly requested in Comment #2) the direct comparison between dLSBM and CBM on the same spheroid samples (N=5) (**new Supplementary Fig. 7**). To quantify the global spatial features of a spheroid, we plotted the Brillouin shift of all pixels into a histogram, which consistently featured two peaks for each spheroid analyzed. Importantly, we have shown that there is no statistical significance between the results of dLSBM and CBM of both peaks (we have updated **Fig. 1e** of the manuscript to reflect these new data/analysis).

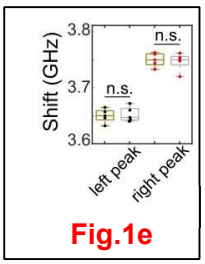


Fig.1e

1F- In a separate experiment (see also **Response 3**), we specifically looked at the biological meaning of the histogram peaks via fluorescence staining of the nucleus (N=14). We show that the right peak of the histogram, which we had showed to be equivalent between dLSBM and CBM, represents the mechanical information of the cell nuclei, a crucial subcellular information of the spheroid (**new Supplementary Fig. 8**).

1G- For the data in Fig.2 (see also **Response 6**), we analyzed the spatial change overtime for spheroids under osmotic shocks in **Fig. 2a-2c** by plotting time-space kymographs (**new Supplementary Fig. 11**), from which we can observe that the spatiotemporal evolution of mechanical features is not uniform across the regions of the spheroids and we can follow the behavior of specific regions of the spheroids.

1H- We appreciate the papers mentioned by Reviewer #1, they included work performed by our group. However, we must note that the Brillouin images therein were acquired by confocal Brillouin with much higher NA (0.6-1.4) compared to the one used in dLSBM (NA=0.3). For a visual comparison, it is probably more informative to compare our data with confocal images of comparable NA (such as: *Mahajan et al, Cancers 13, 5549, 2021; Scarcelli et al, IOVS 55, 4491, 2014*). We chose NA of ~0.3 in this work with the consideration of achieving both cellular resolution and enough FOV for spheroid samples. Higher NA is possible in dLSBM, but it requires changing optical design/components; we hope the Reviewer agrees this is impractical for this revision.

With the new data, analysis and considerations, we hope the reviewer agrees that the image quality of dLSBM and CBM is comparable and both provide spatially-resolved mechanical contrast.

Comment 2: Fig. 1d: The attempt to compare between CBM and LSBM is appreciated, yet some important details are missing: Was the comparison made using the same scattering geometry? similar SNR and Brillouin shift precision? What was the acquisition time per pixel used with the confocal setup? How does the difference in these parameters and the different wavelength used affect the fair comparison between CBM and LSBM? Also, it seems that the sample was not imaged simultaneously, thus multiple independent experiments should be performed to show statistical insignificance between the methods.

Response 2: We appreciate the Reviewer's favorable comment about these comparative measurements and apologize for missing the technical details. We have fixed these shortcomings in the revision:

2A- We have updated the Method section and the caption of Fig.1 to add all the missing information. To briefly answer the questions: the CBM setup works at 180° geometry while dLSBM works at 90° geometry. The CBM is shot-noise limited, acquisition time of 50 ms, and spectral precision of ~10 MHz to ensure a fair comparison. The Brillouin shift ω_B is physically determined by the following equation:

$$\omega_B = \frac{2n}{\lambda} \cdot \sqrt{\frac{M'}{\rho}} \cdot \sin\left(\frac{\theta}{2}\right)$$

Where λ is the laser wavelength, θ is the collected scattering angle, n, ρ, and M' are refractive index, density, and longitudinal modulus of the sample, respectively. Based on this equation, the Brillouin data of CBM with different geometry and wavelength can be readily converted to the case of dLSBM without affecting the accuracy of the results.

2B- As requested, we conducted new experiments to expand the comparison of dLSBM vs CBM. This is a challenging experiment in practice because the samples need to be moved to two different systems (with different mounting procedures, geometries, voxels); and, thus after the imaging, the co-registering of the imaged plane is not straightforward, nor guaranteed to be accurate on the micron-scale. Nevertheless, we agree with the Reviewer that this is an important experiment, so we were able to acquire new data on multiple (N=5) independent spheroids (**new**

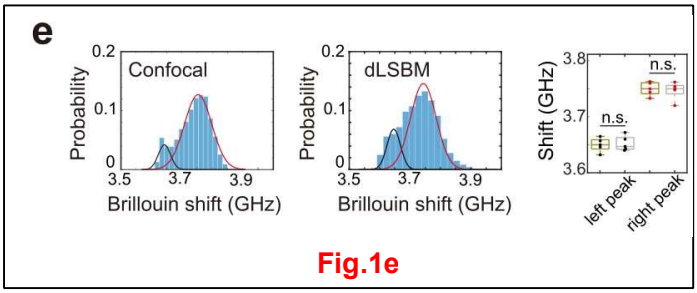


Fig.1e

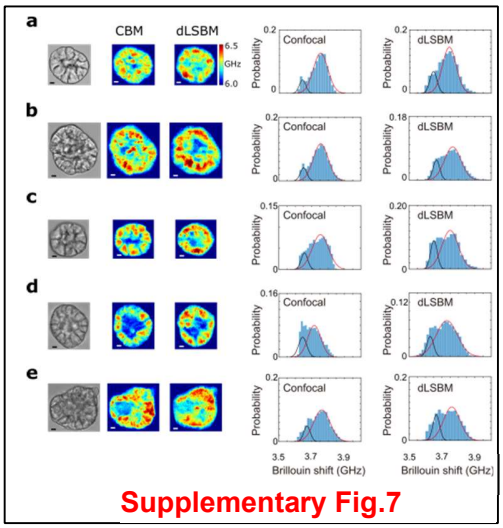
Supplementary Fig. 7). We quantified the spatial features of each spheroid by plotting the Brillouin shift of all pixels into a histogram. The histograms for each spheroid consistently featured two peaks (the stiffest representing the nuclei of spheroid cells as demonstrated in **new Supplementary Fig. 8**). We confirmed that there is no statistically significant difference between the results of dLSBM and CBM (we **updated Fig. 1e** in the main text).

Comment 3: Fig. 1e: What is the origin of the lower frequency Brillouin shift? Was it confirmed statistically in multiple independent experiments as the difference in frequency is around 100 MHz? Could it be confirmed also pixelwise rather than using the statistics from the whole Brillouin image? While two frequency components are observable in the CBM spectrum, their existence is unclear by inspecting only the LSBM spectrum. Why? What are the implications of this latter point on the accuracy of the measured LSBM spectra and the image quality?

Response 3: Apologies for the confusion. We think the reviewer refers to the histogram extracted from the Brillouin images. Here are new data to address this comment:

3A- We have analyzed the expanded comparison of Comment #2 and the histograms for all samples are now shown in **new Supplementary Fig. 7**. Performing careful analysis with a method we have previously validated [*Zhang et al, Lab on a chip 17,663,2017*], we confirmed that:

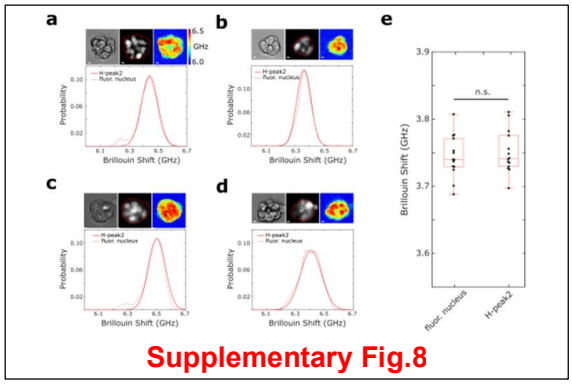
1) for both instruments, the histograms for all samples are best fit by two peaks;
2) there is a strong statistically significant difference ($p<10^{-5}$) between left (lower shift) and right (higher shift) peak;
3) there is no statistically significant difference between the location of the peaks in dLSBM vs CBM (**updated Fig.1e**).



Supplementary Fig.7

At visual inspection of the data in the original manuscript, we agree with the Reviewer that the two peaks were more observable in the CBM histograms we had shown; however, this is not due to a fundamental difference in the two instruments but more to the practical issues detailed in **Response 2B**. The data of independent samples in **new Supplementary Fig. 7** further clarify that there is variety in visual appearances of the histograms between different samples even when taken with the same modality. For this reason, we want to emphasize that we did not use visual impressions to identify peaks and quantify the peak locations, we employed a peak-fitting algorithm that we have validated in a previous paper [*Zhang et al, Lab on a chip 17,663,2017*]. In the revision, to further improve our histogram analysis, we have also upgraded our image processing routine after cross-validation of new theoretical calculations and experiments regarding refractive index behavior (details in **Response 2 to Reviewer #2**).

3B- We collected new data to interpret the biological meaning of the histogram peaks. We have already validated this analysis in single cell imaging [*Zhang, Lab on a Chip 17 (4), 663-670, 2017*] where the two histogram peaks correspond to the cytoplasm and nucleus regions, as the nucleus has higher modulus. We hypothesized that the same interpretation holds for spheroids: to verify this experimentally, we took new data using our available co-registered confocal fluorescence+Brillouin microscope and stained the nuclei of the spheroids' cells (N=14). The overlap with fluorescence image allowed us to extract Brillouin shifts of nuclear region explicitly based on a spatial analysis. The extracted nuclear shifts from spatial analysis are clearly equivalent to the right peak of the histogram (**new Supplementary Fig. 8e**). The left peak of the histogram would require more fluorescent channels for a proper interpretation in spheroids comparing to single cells, because it includes not only cellular cytoplasm but also medium and extracellular matrix between cells. This new set of data answers directly the Reviewer question: the right peak derived from the histogram provides information on the spheroids' cell nuclei and thus, as expected, has consistently higher Brillouin shift values.

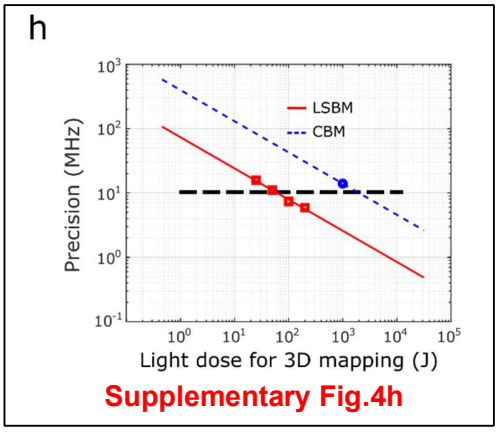


Supplementary Fig.8

Comment 4: Fig. 1g,h: A comparison between CBM and LSBM for sample viability as a function of light dose is important. What is the maximal light intensity in CBM at 780 nm to enable sample viability similar to that of LSBM? What is the best acquisition time per pixel that can be achieved by CBM at this maximal light intensity compared with that of 1 ms? What is the image quality and Brillouin shift at this maximal light intensity and best acquisition time per pixel compared with that of LSBM at 1 ms?

Response 4: Thank you for this important suggestion, which prompted us to perform a comprehensive characterization of the light dose aspect of the paper. To facilitate the explanation of the various parameters we note that:
1) the sample viability is determined by the total light dose (light intensity times total acquisition time);
2) for a given spectral precision, higher intensity will allow shorter acquisition time, and vice versa.
Therefore, the most comprehensive way to answer the Reviewer's question is to compare the light dose of dLSBM and CBM under the same spectral precision. This also allows comparing the spectral precision of two setups under the same light dose.

We characterized all these parameters thoroughly, and here we show a new plot of spectral precision at varying light doses in 3D mapping for dLSBM and CBM under shot-noise limited condition (**new Supplementary Fig. 4h**). (Not having access to a CBM instrument at 780nm, here the CBM data are from state-of-theart published paper by Guck's group). We show that at the same spectral precision (e.g. 10 MHz), CBM needs ~32x higher light dose (black dashed line) than our dLSBM setup (thus more phototoxicity). Similarly, at the same light dose (i.e. same viability), the spectral precision of CBM is 5.4x worse than dLSBM (43 MHz vs 8 MHz).



In practice, the maximal light power/intensity of CBM is decided to make sure the sample is not damaged under long time of illumination, which is fairly established in the literature to be around 10 mW to 60 mW [e.g. *Schlüßler, Biophys J. 2018; Bevilacqua Biomedical Opt. Exp., 2019; Nikolic, Biomedical Opt. Exp., 2019*]. For the same spectral precision and with equivalent laser energy dose to the sample, the best acquisition time of CBM is 32 ms compared to 1 ms of dLSBM.

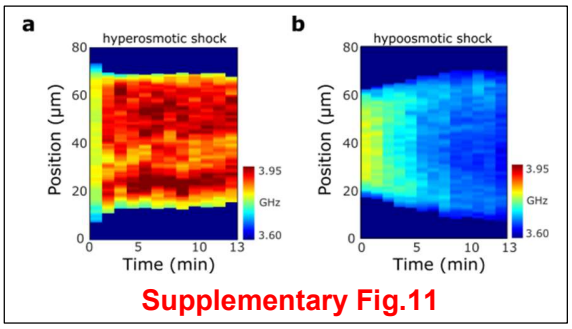
Comment 5: Fig. 2a-c: Why do no spatial features appear in the hypoosmotic sample? Is it due to the domination of water in the sample? Or is it related to the factors mentioned in comment 1 that mask the spatial features?

Response 5: We thank Reviewer1 for this insightful comment. We performed more analysis to elucidate the issue. We excluded that this observation is an artifact due to loss of spatial resolution; indeed, the time-space kymographs for osmotic shocks clearly show observable evolution of spatial features (**new Supplementary Fig. 11**, see **Response 6**). The effect noted by the Reviewer has to do with hydration behavior. On one hand, when water becomes dominant, Brillouin sensitivity to mechanical contrast diminishes. This is well known in our community [*Wu et al, Nat. methods 15, 561,2018; Scarcelli et al, Nat. methods 15, 562, 2018*] and can be quantified: if the initial water content (volume fraction) is ~76% in spheroids [*Delgado et al, PLOS one 8, e67590, 2013*], the final water content under hypoosmotic shock is ~88%, which results in a ~2.5-fold loss in Brillouin sensitivity to solid-network modulus contribution. On the other hand, importantly, this is a true and well expected behavior from polymer theory and tissue experiments because the dependence of elastic modulus on pore size is much steeper at small pore sizes and becomes flatter at large pore sizes *[e.g. Carmona et al, Gels 7, 186, 2021]*. We have edited the manuscript to include this observation of the Reviewer and the related discussion.

Comment 6: Fig. 2d,e and 2g,I,j: While the average and std of the Brillouin shift are important measures in the analysis of spatiotemporal processes, the described analysis lacks the spatial dimension

that is required for a comprehensive understanding of spatiotemporal processes in biological samples. Can spatiotemporal information be provided by LSBM at each pixel (in addition to the radial averaging of the Brillouin shift that adds spatial information but only marginally)? or perhaps LSBM can be principally used to parallelize measurements of processes with dynamics slower than the exposure time of the camera (for single line imaging)?

Response 6: We are confident with the above new data that we have demonstrated that our technique gives access to spatial features. Data presentation is dependent on the readout that will answer the desired goals of a given biological question. If the goal is to follow the evolution of sub-regions of the spheroid, dLSBM is shown here to be capable of doing that, but a co-localized fluorescence staining would be needed for full biological understanding. To address this comment specifically to the studies in Fig. 2, as a demonstration, we plotted the kymographs of spheroids under hyperosmotic and hypoosmotic shocks (**new Supplementary Fig. 11**). To do this, we selected a central line of the spheroid and plotted the Brillouin shift over time, from which one can observe the temporal mechanical change for each pixel. The kymographs show that the change in Brillouin shift is not uniform across the spheroid and instead localized regions of higher/lower shift can be identified. The final suggestion of the Reviewer is also valid: dLSBM can also be used for single line imaging to capture mechanical dynamics slower than the exposure time of the camera.



Supplementary Fig.11

Comment 7: Fig 2g,h: Besides being noncontact, what are the advantages of imaging by LSBM over AFM at some points given that the LSBM images are essentially spatially averaged to obtain useful quantities?

Response 7: We chose to present spatial averages because we thought that was the most appropriate analysis, but this is not a limitation of the technique. To recap, several examples have been provided of the type of spatial feature analysis we can provide that would be impossible with AFM: the identification of stiffer components (cell nuclei) within the spheroids; the radial averaging revealing that the cores of tumor spheroids stiffen; the spatial std analysis revealing the internal homogenization of spheroids in different osmotic conditions; the kymographs indicating the spatial and temporal mechanical change within a spheroid under osmotic shocks. All of these data/analysis are not achievable with any contact or spatially-averaged technique.

With these observations, we have revised the manuscript to highlight that we provide access to microscopic mechanical features, and within the interior of biological samples, i.e. in regions that are not accessible to AFM.

Comment 8: How was the acquisition time per pixel of 1 ms in the spheroids selected? It seems that both the spheroid and water samples were measured with the same acquisition time per pixel of 1 ms; Was the SNR and Brillouin shift precision obtained in the spheroids similar to those measured in water although spheroids represent a complex medium while water a simple fluid? A methodological way for choosing the appropriate acquisition time in biological samples is missing.

Response 8: This is an important point. The "Bio-Brilllouin" field has published an opinion paper identifying the need for standardization [*Antonacci et al, Biophysical Reviews, 2020*]. Water is generally the sample of choice in this community because of the relative abundance of water in biological samples and the ease of performing standardized measurements. Therefore, we have used water as primary sample for characterization. However, the Reviewer makes a good point, we need to show performances in our biological sample under test. To address this comment, we have characterized the spectral precision in tumor spheroids and shown it is shot-noise limited (**new Supplementary Fig. 4g**). With the same light dose, the spectral precision (also SNR) in spheroid is slightly worse than in water (~1.2x laser power is needed for spheroid to achieve the same precision as in water). The 1 ms acquisition time was chosen to achieve spectral precision of 10 MHz while the input laser energy does not cause any photodamage. We have expanded the Method section (*Characterization of dLSBM setup*) to explain how the acquisition time is chosen for biological sample.

Comment 9: Supplementary Fig. 3: It seems that there is a dependence of the spectral precision/resolution on the spatial position in the sample. Study of this point and its implication on the accuracy and precision of the Brillouin spectrum measurements should be performed.

Response 9: Indeed, the laser intensity distribution along the illumination axis changes due to the light propagation in the sample, which will affect the spectral precision. We have quantified the precision of the measurement along different positions of the illumination axis (**new Supplementary Fig. 4d**). As can be seen, most of the pixels have precision of <10 MHz, and pixels close to the edges have slightly worse precision (<13 MHz). The average precision of the whole illumination line is 9.8 MHz.

Minor comments:

Comment 10: What are the cellular components shown in Fig. 1c, 2a-c, and 2f? How many pixels were recorded for a single image? What is the maximum frame rate experimentally achieved by LSBM?

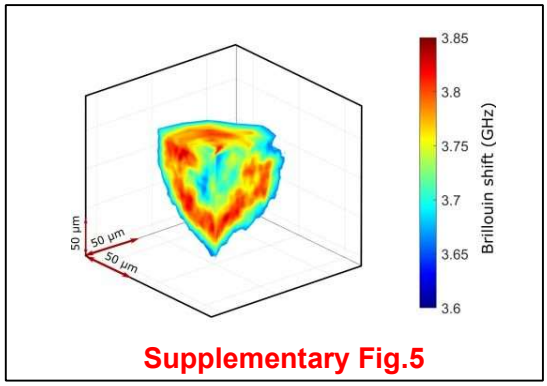
Response 10: Through previous responses and new data (Response #3, **new Supplementary Fig. 8**), we have confirmed that regions of higher Brillouin shift are generally dominated by cellular nuclei, and the rest are probably a combination of cytoplasm, medium, and ECM. We note, though, that to

identify specific cellular behaviors one would ultimately need a co-located fluorescence light-sheet microscope, which was not available in this work.

For a single image, 100*200 pixels were recorded, and this takes 20 seconds, which is the maximum frame rate used in this work. We have added this information in the caption of the figures.

Comment 11: Volume rendering of the image data set in Fig. 1c would be essential to clearly visualize the sample in 3D.

Response 11: We thank the reviewer for this comment. We have performed 3D rendering of Fig.1c (**new Supplementary Fig. 5**) which allows readers to visualize the sample in 3D.



Supplementary Fig.5

Comment 12: It seems that the scale bar information for Fig. 2f is missing in the caption.

Response 12: We thank the Reviewer for pointing this out, we have added this information in the caption of Fig.2.

Comment 13: How were the Bragg and Fabry-Perot filters stabilized with respect to the laser line?

Response 13: The Bragg filter is very stable and needs little adjustments over long term (~ several months), the FP filter has a slow drift that requires optimization over time (~ hour). For long term stabilization, a feedback loop can be built to correct the drift in real time. We plan to implement this in our setup in the future. We have revised the manuscript to include this information.

Comment 14: It seems that there is a typographical error in the x-label of Supplementary Fig. 3a,b and it should be “spectral axis”.

Response 14: We have corrected this typo in the updated Figure. Thanks for the reminder.

Comment 15. A title should be added to the color bar in Supplementary Fig. 3a

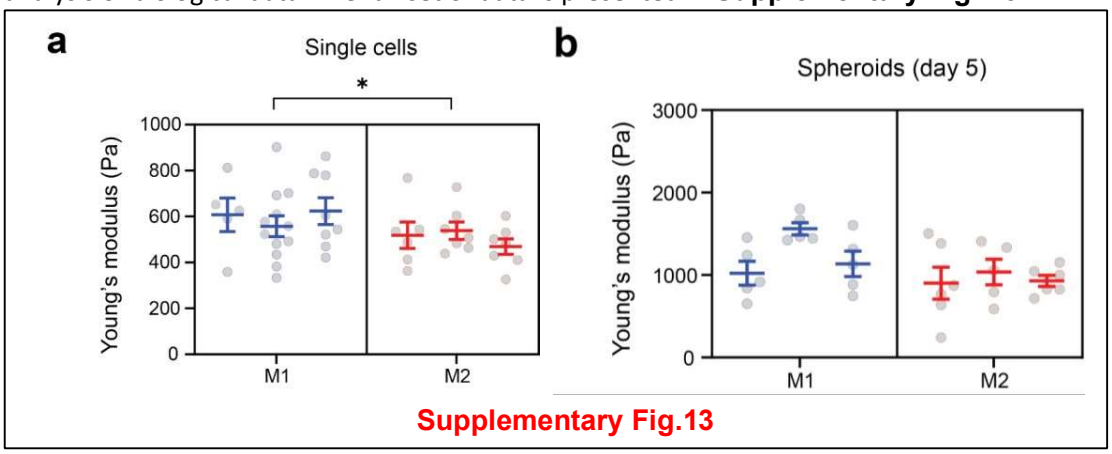
Response 15: We have added a title (i.e., *Photon number*) to the color bar.

Comment 16. For clarity and consistency with Supplementary Fig. 3c, the spectral axis of Supplementary Fig. 3a,b should be presented in GHz. Are the counts in the y-axis of these figures represent electrons or photons? having these counts in photon numbers would be clearer.

Response 16: We thank the Reviewer for pointing these issues. We have added GHz label to Supplementary Fig. 3b (now **Supplementary Fig.4b**), from which one can convert pixels to frequency. We also converted the counts into photon number.

Comment 17. The sample size of the AFM measurements is significantly lower than that of the Brillouin measurements. What is the effect of this difference on the statistical analysis/interpretation of the data?

Response 17: As a matter of fact, also with AFM we measured ~20 samples per condition (similar to dLSBM sample size); however, AFM data were taken as three culture/measurement sessions; therefore, they are treated as repeats, i.e. N=3, for analysis of the experiment. This follows the statistics guidelines for the analysis of biological data. The full set of data is presented in **Supplementary Fig. 13**.



Supplementary Fig.13

Comment 18. Was the stated optical resolution measured? If not, this should be indicated explicitly.

Response 18: As shown in Response 1, we have conducted a comprehensive characterization of the spatial resolution (**new Supplementary Fig.6**).

Comment 19. Was shot-noise operation confirmed experimentally in the spheroids?

Response 19: As shown in Response 1, we have confirmed the shot-noise limited operation in the spheroid (**new Supplementary Fig.4g**).

Comment 20. What is the purpose of the green light and O5 in Fig. 1b?

Response 20: We apologize for missing this information. The green line and O5 is a home-build brightfield microscope for image guiding and alignment of dual line. We have added the description in the caption.

Comment 21. For clarity, the spatial and spectral axes should be denoted on the camera plane in Fig.1b **Response 21**: We have labelled the spatial and spectral axes on the camera plane in updated Fig.1b.

Comment 22. What was the spectral resolution of the VIPA spectrometer?

Response 22: As mentioned in Response 1, the spectral resolution of the VIPA spectrometer is ~0.25 GHz. We have added this additional data to the figure (**new Supplementary Fig.4f**).

Reviewer #2:

Comment 1: A Summary of Key Results: The Brillouin microscope reported here uses line scanning from opposite directions and orthogonal detection to increase image speed to 1 ms per pixel and reduce dose rates to 7.4% of what would be required for conventional confocal point scanning. It achieves this based on spatial resolution of 1.5 µmx1.5 µmx4.8 µm, whilst achieving spectral extinction >70 dB and spectral precision of <10 MHz.

Could the authors also please specify the field of view of their microscope? And can they also ensure FOV and voxel number is included or able to be readily deduced in each experimental case, as well as the total acquisition time. (At bottom of p2 Supplementary an (axial?) FOV of 150-300 □m variable is mentioned.)

The key results in this work are to show that Brillouin microscopy is viable on live spheroids over a time lapse of five days, and to demonstrate short term changes can induced by osmotic shock can be imaged, every minute over 13 minutes.

Response 1: We thank the Reviewer for this comment. Similar to light-sheet microscopy, the FOV of the dLSBM is mainly determined by the length of the illumination beam. We can achieve a FOV of ~150 µm using single line illumination, and this can be extended to as much as 300 µm under dual-line illumination. For experiments of this work, the scanned volume is a cylinder, and the typical FOV we can achieve is 150 µm x 150 µm x 160 µm. We have added the information of FOV and acquisition time for each experimental case in the main text and captions of figures.

Comment 2: B Originality and significance: Line scanning in an orthogonal detection geometry was proposed by this group and published in 2016 – Sci Reports: Ref 25. To this geometry is added a counterpropagating illuminating beam and an elegant explanation of how the droop in Brillouin frequency with depth in the sample can be compensated. How do the authors justify this work relative to their 2016 work, and how do they rebut the claim of incremental improvement? And can the authors please reference the state-of-the-art they claim to improve upon (lines 67/68).

Overall, it is indeed encouraging to see Brillouin microscopy continue to progress as a non-contact mechanobiological imaging technique – I am supportive of publication, once minor issues are addressed and subject to a convincing explanation of novelty relative to Sci Rep 2016.

Response 2: Our Sci Rep 2016 paper was a proof of principle that would not be capable to measure biological samples. In the past six years we have made dramatic technical upgrades to make the instrument viable for biological research applications; they can be summarized/quantified as follows:

1. 1000-fold increased spectral extinction, reaching the 70 dB needed for biological samples.
2. Over 80-fold reduction in absorption-induced photodamage of 780nm vs 532nm wavelength.

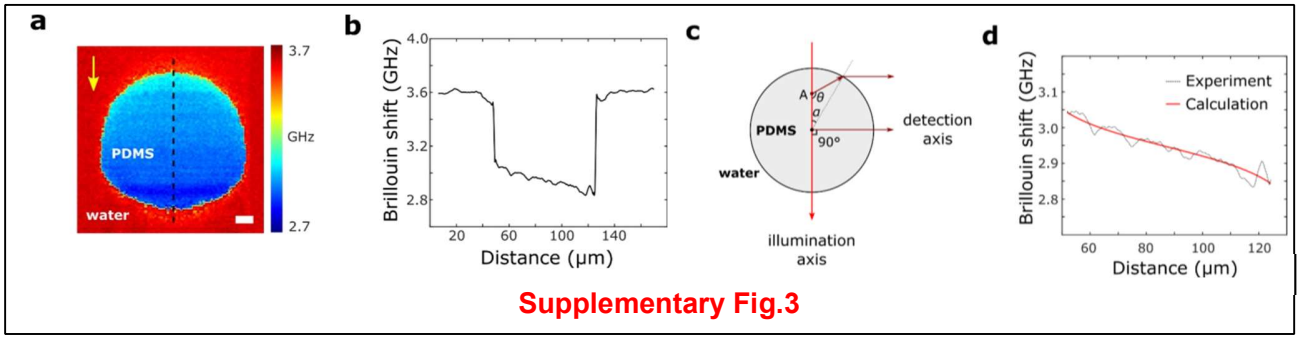
3. Dual-line illumination, which enabled correcting refractive index artifacts and optical distortion, which is critical in biological samples.
4. Laser stabilization based on atomic transition, which enabled longitudinal biological studies.

Here are more detailed explanations for each of these characteristics:

1. Our 2016 work used a 532 nm laser source, and the overall extinction of the spectrometer was 35 dB, which is not able to measure biological samples due to the large non-Brillouin scattering components. In this work, we locked a 780 nm tunable laser to a Rb absorption line and used a Rb gas cell as a notch filter to improve the spectral extinction to >70 dB, allowing us to measure biological samples in shot noise limited operation.

2. The longer wavelength of the laser source also provides us with a great advantage of phototoxicity. While Brillouin scattering *per se* does not induce photodamage, the corresponding absorptioninduced photodamage is strongly wavelength-dependent and can be reduced 80-fold when switching wavelength from 532 nm to 780 nm, as we showed in [*Nikolic, Biomedical Opt. Exp., 2019*]. This is crucial for longitudinal measurement of the tumor spheroids.

3. The dual-line illumination is critical for imaging biological samples because it enables correcting the refractive index induced artifacts. To demonstrate the importance of this aspect of this paper, we performed and reported a new detailed study on the artifacts caused by the refractive index mismatch using a PDMS sphere immersed in water. As shown below, our theoretical calculations are in good agreement with our new experiments. We have updated the manuscript with this new dataset and analysis (**new Supplementary Fig.3** and updated **Supplementary note1**), which also helped optimizing the postprocessing of dual-line illumination data (see also **Response 4**). The dual line illumination also enabled expanding the FOV of the Brillouin microscope.



Supplementary Fig.3

4. Finally, a practically important improvement of this work is the long-term wavelength stability (~MHz-level) of the setup. The 780 nm laser allowed us to lock its frequency to the absorption line of Rb gas thus enabled longitudinal biological studies with an absolute frequency reference. Since the 532 nm laser of our 2016 work does not rely on absolute frequency stabilization, longterm experiments were very challenging and calibration procedures, usually based on reference materials, were imperfect due to the dependence on environmental conditions.

To specifically address the last question of this Reviewer comment, we have cited two references of the state-of-the-art that we compare with: (1) *Remer et al, Nature methods 17, 913 (2020)*, which reported stimulated Brillouin microscopy with acquisition time of 20 ms in water sample; (2) *Antonacci et al, Biophys. Rev. 12, 615 (2020)*, a review paper which summarized the state-of-the-art confocal Brillouin microscopy which has acquisition time of 20 ms – 200 ms.

Comment 3: C Data & methodology: Mapping the multiple voxels axially along the line of illumination onto the spectrometer is termed multiplexing, which is a term that is widely used in other spheres. In particular, as regards simultaneous counter-illumination, it becomes confusing as to whether both beams illuminate simultaneously, or how each beam are sequentially switched on. Can the authors make this very clear throughout please. Can the authors briefly outline (perhaps in Supplementary materials) the extent to which the multiplexed spectrometer performance is compromised (or not) by the multiplexing. Can the wavelength of the laser implicit in using a Rb cell be explicitly stated when introduced please.

Response 3: This is an important point; we have modified the main text and Method section to detail how the dual-line illumination works: each beam is sequentially switched on by a flip mirror (**Supplementary Fig.1a**). For multiplexed spectrometer, the propagation property of Gaussian illumination beam causes the trade-off between multiplexed number and the axial resolution. This tradeoff however could be mitigated by using different illumination strategies (e.g, focus extension, Bessel beam, etc). We have added the above discussion at the end of **Supplementary note 1**.

We apologize for missing the wavelength information of the laser when talking about the Rb cell. We have added this to the main text as follows: '*… locking the laser frequency (780.24 nm) to a Rb-85 absorption line (D2 hyperfine structure)*'

Comment 4: Whilst the droop in Brillouin frequency versus depth is a very interesting feature, most Brillouin measurements are relative rather than absolute – can a discussion of this point be included please. For example, under time lapse imaging as shape changes then intrinsic shift will change independent of local biomechanics. As well, time lapse at a single point implies looking for changes not absolutes. In general, this rather critical point should be further elaborated.

Response 4: This is an important comment. If the biomechanical information of specific locations is of interest, as a spatial comparison or as an evolution over time, the gradient due to the refractive index mismatch needs to be considered carefully. Thanks to our new study of the refractive index behavior (detailed in **Response 2**, new **Supplementary Fig. 3** and updated **Supplementary note1**), we designed a procedure to estimate and correct artifacts due to refractive index mismatches, which works well for spherical objects such as spheroids. For biological sample with irregular shape, 3D mapping of the refractive index will be required for an accurate correction. Besides the noted new data/analysis, we have updated the main text and supplementary note to emphasize this important discussion.

Comment 5: E Conclusions: robustness, validity, reliability: Overall, this manuscript provides a modest but sufficient level of validation of its key findings and provides most but not all key experimental details as commented on above.

Response 5: We thank the Reviewer for this comment. During the revision, we have collected several new datasets for validations and characterization (more details are available in the response to Reviewer 1). For example:

1. We conducted comprehensive characterization of the spatial resolution, spectral resolution, SNR, shot-noise limited operation with both test sample and spheroid (**new Supplementary Fig. 4 & 6**).
2. We expanded the direct comparison between dLSBM and CBM by measuring multiple independent spheroids and validated the biological meaning of the histogram peak (updated **Fig.1e, new Supplementary Fig.7&8**) to provide nuclei-specific quantification.

We hope these new datasets and discussion strengthen the level of validation of our technique.

Comment 6: F Suggested improvements: experiments, data for possible revision: Mention is made of the use of a tunable lens to further reduce the illumination intensity five-fold – obviously doing this would be a worthwhile exercise, but I would not see this as mandatory here.

Response 6: We thank the Reviewer for understanding that introducing a tunable lens is beyond our capability during this revision.

Comment 7: H Clarity and context: I found the abstract rather weak and did not convey the key novelty and achievements well at all.

Response 7: We have revised the abstract to try to better capture the main concepts of the paper.

Reviewer #3:

Remarks to the Author:

This paper from Zhang et. al. presents an improved multiplexed Brillouin microscopy technique based on line-scanning and single-shot spectral analysis. The authors expand a previously developed linescanning setup (Zhang et. al., Scientific Reports 2016) by equipping it with a dual-line illumination that enables artifact-free imaging of thicker samples. Importantly, the new method greatly improves the speed of acquisition allowing fast 2D/3D imaging of biological samples with minimal photodamage. This is supported by several experiments demonstrating the capabilities of LSBM for mapping mechanical properties of living tissue: the authors demonstrate 3D Brillouin mapping of spheroids and characterize sample viability and proliferation following irradiation. Furthermore, they show that the method can be used to capture dynamic changes in tissue mechanical properties by imaging spheroids after a hyper/hypo-osmotic shock and comparing the evolution of mechanical properties in healthy and tumor spheroids over time.

Overall, the new method presented here by Zhang and colleagues overcomes current limitations of Brillouin microscopy that have hindered its wide application in mapping mechanical properties of living tissues and will be of great interest to a wide community. I therefore recommend publication once the points below are addressed:

Major points:

Comment 1: Since the speed of acquisition is one of the main strengths of the new method, it would be helpful to refer to the overall acquisition times more explicitly in the text. For example, it would be informative to compare the overall acquisition times of the images in Figure 1 panels c and d. This will allow readers to grasp more easily what spatio-temporal scales can be imaged with the presented method. In the same way, the choice of illumination time for the photodamage experiment should be justified (would 5 minutes be a typical acquisition time?).

Response 1: This is an important suggestion. To answer the Reviewer's question, for a typical 2D frame (100x200 pixels), the acquisition time is about 20 seconds while the equivalent frame with confocal setup would take ~16 minutes; for 3D mapping, including 10 frames at different sample rotation angles, the total acquisition time for dLSBM is less than 4 minutes (the total illumination time is about 3 minutes, this is why we chose 5 minutes in photodamage experiment), while for confocal setup with similar spectral precision, it will take more than 2 hours. We have added the acquisition time and total number of pixels for each experiment in Fig.1 and 2, we also provided the information of FOV of the setup and added the justification of photodamage experiment in the main text and the Method section.

Comment 2: The negative control used in Figure 1g should be specified in the text / Figure legend together with its N number.

Response 2: We apologize for missing this information. The negative control was a single experiment used for sanity check that the dye of Ethidium Homodimer-1 (red channel) works. To prepare the sample of dead spheroid, we removed the medium and added -20º C methanol for 15 min. We have added this information to the caption of Fig. 1 and the Method section.

Comment 3. The statement that authors are able for the first time to observe stiffening of growing tumor spheroids, guided by a stiff core is perhaps slightly overstated. It would be better to rephrase this part (lines 154-156), putting it in the context of several studies looking at mechanical properties of tumors organoids/spheroids that have previously highlighted the presence of a stiffer "core" (Han et al, Nature physics 2020, Mahajan et al, Cancers 2021).

Response 3: We thank the Reviewer for the suggestion of these studies, which help place our work in context. We have cited these studies and revised the manuscript accordingly. Now it reads as:

'*Consistent with previous studies*[28, 36]*, we observed a robust stiffening behavior in the evolution of growing tumor spheroids which we validated against gold standard AFM*.'

Comment 4: In the last section, authors could further expand the discussion of strengths and limitations of the technique: for example, lines 145-149 are quite vague ("analysis of different sample types", "different mounting configurations"). We would encourage the authors to be more explicit in this discussion as it will allow a broader readership to evaluate the applicability of the method presented to different systems and questions of interest (some interesting points could be a discussion of the size/thickness of samples, possibility to image developing tissues/embryos, combining the system with other imaging modalities like fluorescence).

Response 4: These are all valuable and agreeable suggestions. We have expanded the discussion (mindful of the length limits of the brief report format) to address the strengths and limitation for future practical use explicitly. Here's the revised paragraph:

'*Several improvements of the technique could be implemented from advances known in light-sheet fluorescence microscopy: e.g. the efficiency of the technique could be improved by axially scanning the focus of the illumination beam; changing the configuration from horizontal-view to inverted-view will make the sample preparation easier and more flexible; the field of view could be improved for large sample by extending illumination beam's depth of focus; the penetration depth could be increased by the adaptive optics techniques; and the combination of dLSBM and light-sheet fluorescence microscopy will allow coregistered Brillouin and fluorescence imaging*.'

Minor points:

Comment 5: Line 46, the references provided for examples of mechanically-relevant in vivo processes are somewhat narrow in scope, it would be useful to add references to a more extensive discussion of the topic (eg reviews).

Response 5: We thank the Reviewer for pointing this out. We have added the following two comprehensive review papers as references to broaden the discussion of the topic.

22. *Butler, D.L. et al. The impact of biomechanics in tissue engineering and regenerative medicine. Tissue Engineering Part B: Reviews **15**, 477-484 (2009).*
23. *Gilmour, D., Rembold, M. & Leptin, M. From morphogen to morphogenesis and back. Nature **541**, 311-320 (2017).*

Comment 6: We would encourage the authors to consider sharing the code developed for this project on an open-source platform such as GitHub, instead of making it available upon request. This is especially true for the image fusion code which could be helpful to other researchers in the community interested in adopting a dual-line illumination scheme.

Response 6: This is a great suggestion; we will share the codes related to the paper.

Decision Letter, second revision:

Our ref: NMETH-BC48348A

4th Nov 2022

Dear Giuliano,

Thank you for submitting your revised manuscript "Rapid biomechanical imaging at low irradiation level via dual line-scanning Brillouin microscopy" (NMETH-BC48348A). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Methods, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

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Thank you again for your interest in Nature Methods Please do not hesitate to contact me if you have any questions.

Best regards,
Nina

Nina Vogt, PhD
Senior Editor
Nature Methods

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Reviewer #1 (Remarks to the Author):

While the revision address adequately the characterization of the setup, one important concern remains: the spatial resolution. The illumination volume is large here due to the low-NA objective (0.1). The collection NA is also relatively low (0.3). These NA limit the spatial resolution (particularly in the z direction) and hence the ability to discern between mechanical differences in the sample. This ability is further reduced due to the much larger spectral broadening (https://doi.org/10.1063/1.4836477) and the larger phonon wavelength in the 90-degree scattering geometry of the setup as well as the relatively low spectral resolution of the spectrometer. These factors are not addressed in the manuscript but should have been as they would likely affect the capability of the method to acquire subcellular mechanical information in important biological systems (e.g., cells or fine structures of organisms) where sub-micrometer level spatial resolution is required. The spatial averaging of all these factors is clearly seen for example in Supplementary Figure 5 and in Figure 1.1D where one could only speculate what would be the result in cells or organisms using high collection NAs. Moreover, the spatial details observed in the pixels of Supplementary Figure 11 (a) and (b) are weak. I therefore think that claims for high 3D resolution, which are not supported experimentally in this work, should be omitted. Alternatively, experimental evidence for the

appropriateness of the method in biological cells or fine structures of organisms using high NA objectives should be provided. Given the existing experimental data, it seems that the proposed method is appropriate for longitudinal experiments in samples where the 3D resolution is not critical and where spatial averages of the Brillouin shift are sufficient for the interpretation of the experimental results. The Editor will evaluate the fit of the manuscript to the high profile, broad interest journal like Nature Methods.

Reviewer #2 (Remarks to the Author):

Thank you for your clarifications and additions addressing the comments of the three reviewers. My comments have been adequately dealt with and, overall, the manuscript is much clearer for these revisions. A good proof-read for some English grammar, e.g., use of folds instead of fold for X-fold increase, is needed.

Reviewer #3 (Remarks to the Author):

The revised manuscript by Zhang et. al. has been substantially improved with new supporting data/analyses that further clarify the performance and potential of dLSBM, putting it into the context of previous proof-of-principle work by the group and confocal Brillouin microscopes. All major reviewers' concerns have been addressed and we therefore recommend the paper for publication.

I would recommend they add a reference to Prevedel's preprint of a similar technique https://www.biorxiv.org/content/10.1101/2022.04.25.489364v1.full.pdf

And comment on similiarities/differences.

Author Rebuttal, second revision:

Response to comments

We thank the reviewers for the appreciations of the revised manuscript. Below are the detailed point-by-point responses to the additional comments from the reviewers.

Reviewer #1:

Remarks to the Author:

While the revision address adequately the characterization of the setup, one important concern remains: the spatial resolution. The illumination volume is large here due to the low-NA objective (0.1). The collection NA is also relatively low (0.3). These NA limit the spatial resolution (particularly in the z direction) and hence the ability to discern between mechanical differences in the sample. This ability is further reduced due to the much larger spectral broadening (https://doi.org/10.1063/1.4836477) and the larger phonon wavelength in the 90-degree

scattering geometry of the setup as well as the relatively low spectral resolution of the spectrometer. These factors are not addressed in the manuscript but should have been as they would likely affect the capability of the method to acquire subcellular mechanical information in important biological systems (e.g., cells or fine structures of organisms) where sub-micrometer level spatial resolution is required. The spatial averaging of all these factors is clearly seen for example in Supplementary Figure 5 and in Figure 1.1D where one could only speculate what would be the result in cells or organisms using high collection NAs. Moreover, the spatial details observed in the pixels of Supplementary Figure 11 (a) and (b) are weak. I therefore think that claims for high 3D resolution, which are not supported experimentally in this work, should be omitted. Alternatively, experimental evidence for the appropriateness of the method in biological cells or fine structures of organisms using high NA objectives should be provided. Given the existing experimental data, it seems that the proposed method is appropriate for longitudinal experiments in samples where the 3D resolution is not critical and where spatial averages of the Brillouin shift are sufficient for the interpretation of the experimental results. The Editor will evaluate the fit of the manuscript to the high profile, broad interest journal like Nature Methods.

Response: We thank the Reviewer for agreeing that the characterization of the dLSBM setup was adequately addressed in the previous revision. The comment on the spatial resolution of the setup is well received. As we have clarified in the previous revision and response, our setup has diffraction-limited spatial resolution with NA=0.3. This numerical aperture was chosen with the consideration of achieving both cellular resolution and enough FOV for spheroid samples. Higher NA is possible for dLSBM but requires substantial changes of optical design, which is beyond the scope of this work. As a response to this comment, we have replaced 'high resolution' with 'cellular resolution' in this revision to avoid further confusion.

Reviewer #2:

Remarks to the Author:

Thank you for your clarifications and additions addressing the comments of the three reviewers. My comments have been adequately dealt with and, overall, the manuscript is much clearer for these revisions. A good proof-read for some English grammar, e.g., use of folds instead of fold for X-fold increase, is needed.

Response: We thank the Reviewer for the appreciation of the revised manuscript. We have gone through the manuscript to correct the grammar mistakes pointed out by the reviewer.

Reviewer #3:

Remarks to the Author:

The revised manuscript by Zhang et. al. has been substantially improved with new supporting data/analyses that further clarify the performance and potential of dLSBM, putting it into the context of previous proof-of-principle work by the group and confocal Brillouin microscopes. All

major reviewers' concerns have been addressed and we therefore recommend the paper for publication. I would recommend they add a reference to Prevedel's preprint of a similar technique https://www.biorxiv.org/content/10.1101/2022.04.25.489364v1.full.pdf and comment on similiarities/differences.

Response: We thank the Reviewer for recommending the publication of this work. We will discuss with the editor about the proper reference to the similar work from Prevedel's lab.

Final Decision Letter:

Dear Giuliano,

I am pleased to inform you that your Brief Communication, "Rapid biomechanical imaging at low irradiation level via dual line-scanning Brillouin microscopy", has now been accepted for publication in Nature Methods. Your paper is tentatively scheduled for publication in our April print issue, and will be published online prior to that. The received and accepted dates will be February 9th, 2022 and February 13th, 2023. This note is intended to let you know what to expect from us over the next month or so, and to let you know where to address any further questions.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Methods style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

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Please feel free to contact me if you have questions about any of these points.

Best regards,
Nina

Nina Vogt, PhD
Senior Editor
Nature Methods