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Spectrometer Application Report

Mapping the Mechanical Properties of Cells via Brillouin Spectroscopy

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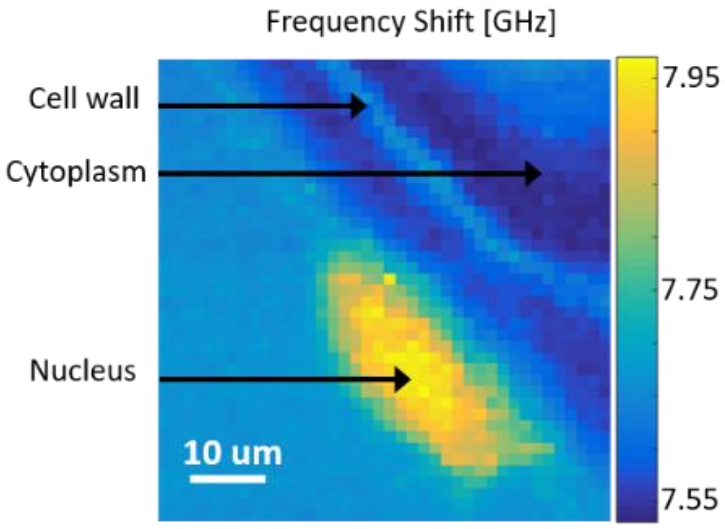

Objectives of this technical note:

- *Optically probing the mechanical properties of biological materials with unparalleled sensitivity.*
- *Demonstrating the high contrast of the Hyperfine Brillouin spectrometer via the ultrafast acquisition of 2D Brillouin maps.*
- *Determining the precision on the measured Brillouin peak positions.*

Background. Brillouin spectroscopy is an innovative tool to study mechanical properties in biology, among other applications. This technique analyzes the inelastic scattering of light following its interaction with acoustic phonons in the sample (interaction between electromagnetic wave and density wave). The resulting energy exchange shifts a fraction of the probing photons by a few picometers. Brillouin signal detection is challenging due to this very small wavelength shift, but also because a negligibly small fraction of the photons is shifted. Elastic and viscoelastic properties can be extracted from the measured Brillouin peak shift and width. Since the technique is contactless, label-free, and offer a micrometric resolution, it holds a promising future for biomedical research and diagnostic.

A 2D map of Brillouin frequency shifts is a powerful tool to better understand how elastic properties are changing across a sample. This can be achieved by scanning the sample point-by-point to extract a series of spectra with their corresponding X and Y positions. The ability to capture small changes in mechanical properties via mapping depends on the precision of the measured peak position. Figure 1 presents a basic scheme of the factors influencing such precision. In the context of biological samples, the changes in frequency shift are often extremely small e.g., ~ a few tens of MHz. A precision of a few MHz is therefore required.

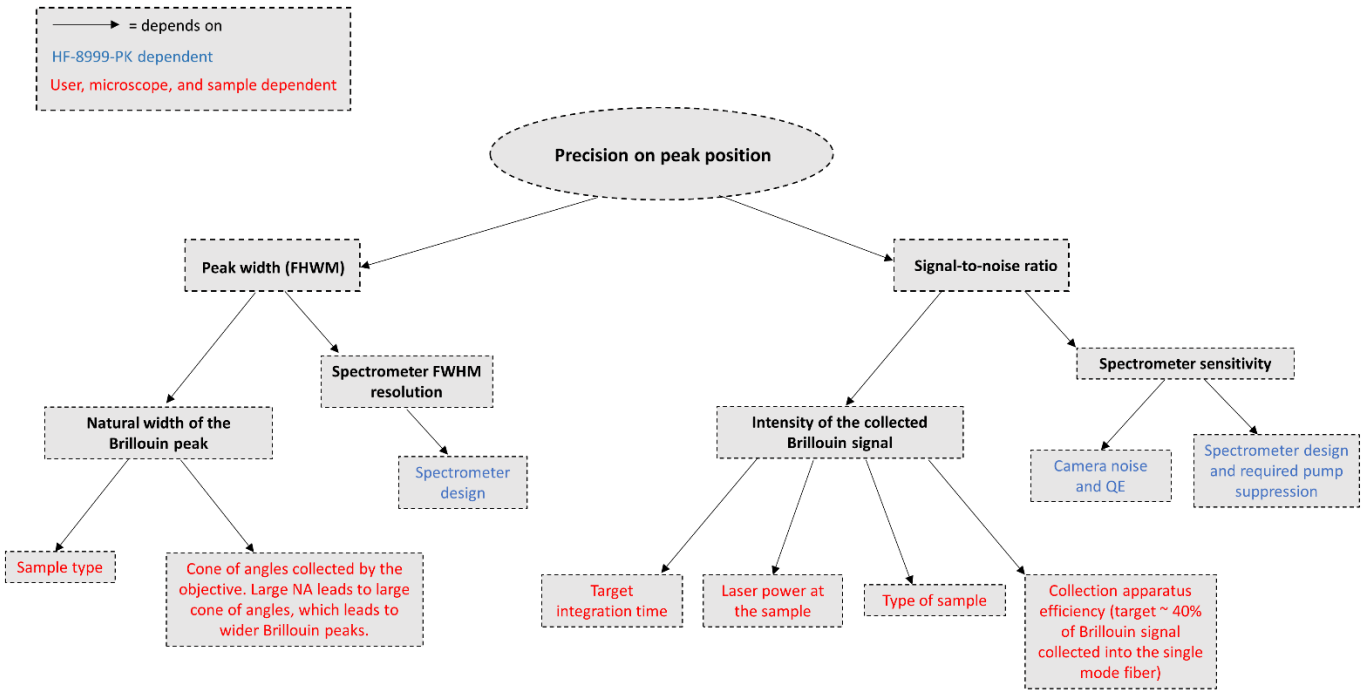

Figure 1: Basic factors influencing the precision on peak position.

Figure 1 shows that the precision depends on both the signal-to-noise ratio and the full width half max (FWHM) of the peak.

Peak width:

In order to limit the influence of the system on the peak width, the FWHM resolution of the spectrometer must be smaller than the natural width Brillouin peaks. Since this natural width depends on both the sample type and the objective NA, selecting the right spectrometer resolution depends on both the application and the specificity of the excitation/collection system. There is an important tradeoff to consider when selecting the objective NA. On one hand a small objective NA reduces the cone of angles collected, thus leading to narrower Brillouin peak and higher precision on the spectral peak position. On the other hand, a small NA produces a large laser spot size on the sample, thus decreasing the spatial resolution (which impact the ability to resolve the small structural features of the sample).

Signal-to-noise ratio:

The spectrometer is designed to maximize sensitivity via i) the high throughput of the unique VIPA/echelle grating configuration, ii) the low insertion loss and 60 dB laser line suppression of the Pump Killer, and iii) the low noise and high quantum efficiency of the camera sensor. While the spectrometer sensitivity is of utmost importance, the intensity of the Brillouin signal that is fed to the spectrometer is also a crucial parameter. The relationship between laser power or time exposure with the Brillouin signal intensity is generally approximately linear. The intensity

of the Brillouin signal also depends on the type of sample under investigation. Finally, the efficiency of the collection system is key; it is generally recommended to couple into the microscope output single mode fiber at least 40% of the Brillouin signal collected by the objective.

Experiment. The sample is excited with a focused single longitudinal mode laser at 660nm (Cobolt Flamenco) using a 20X objective. The laser power at the sample position is approximately 50 mW, unless specified otherwise. The same objective is employed to collect the Brillouin signal in a backscattering geometry. The Brillouin spectra are excited, collected, and analyzed using the Brillouin Hyperfine spectrometer (HF-8999-PK-660) and the Brillouin confocal microscope (HF-9000). Figure 2 presents the experimental setup.

The Brillouin confocal microscope comprises an inverted research grade Nikon Ti2 Eclipse, a motorized Prior Scientific stage, and a Brillouin circulator module that enables confocal excitation/collection. The Brillouin Hyperfine spectrometer comprises a Pump Killer module to suppress the unshifted laser line (~ 60 dB suppression) while transmitting the Brillouin signals (usually shifted by only a few GHz) to the spectrometer module. The latter is composed of a VIPA to disperse the Brillouin spectrum in the vertical direction, providing high resolution but overlapping the orders. An echelle grating separates the overlapping orders in the horizontal direction and enhances the contrast. Using this strategy, 0.25 pm of FWHM resolution can be achieved. The spectrometer used in this study has a resolution of 0.6 pm, or around 0.4 GHz at 660 nm. The resulting 2D light pattern is captured by a Hamamatsu Orca-Fusion CMOS sensor and then converted into a linear spectrum.

The high-resolution motorized stage scans the sample, thus enabling the acquisition of a spectrum for different points on the sample. 2D and even 3D Brillouin maps can thus be constructed from the peak shifts, widths, and amplitudes.

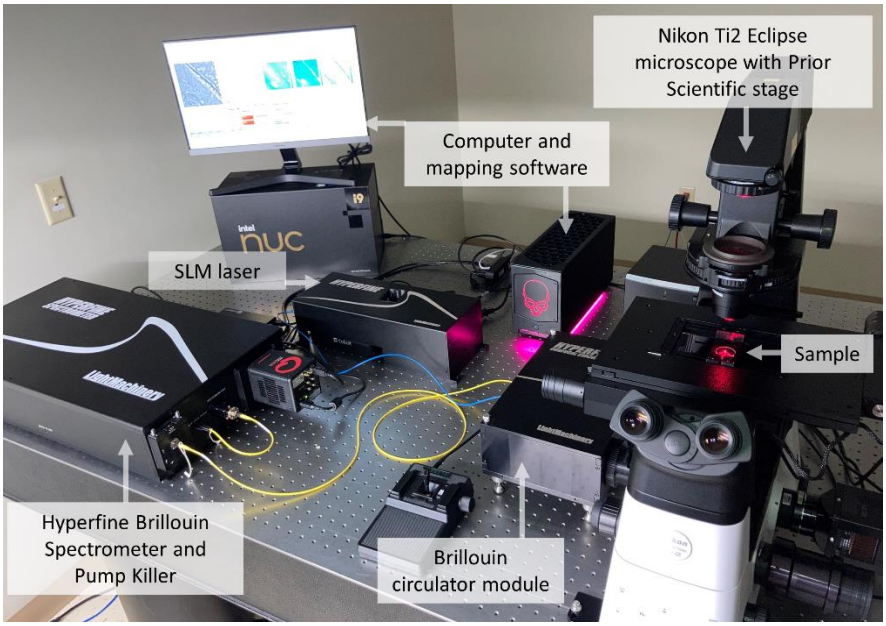

Figure 2: Experimental setup for Brillouin mapping. The single longitudinal mode laser, the Brillouin circulator module, the Hyperfine spectrometer, and the Pump killer are fiber coupled. The Brillouin circulator module is coupled to the Nikon microscope via the left camera port.

Results.

i) Initial testing: sharp glass corner encapsulated in epoxy glue

A proof-of-concept experiment was initially performed to investigate the system's capabilities. A cube of silica glass was polished to obtain a very sharp corner. It was then encapsulated in epoxy glue and cured overnight. Figure 3 presents the Brillouin maps produced by the epoxy. The peak amplitude map indicates the presence of epoxy. The boundaries of the glass cube observed in the brightfield image perfectly match those of the peak amplitude map, as demonstrated by the red dashed lines. In contrast, we see features outside the glass cube in the two other maps. More precisely, the Brillouin shift and FWHM maps indicate the presence of strain in the epoxy near the boundaries of the glass cube.

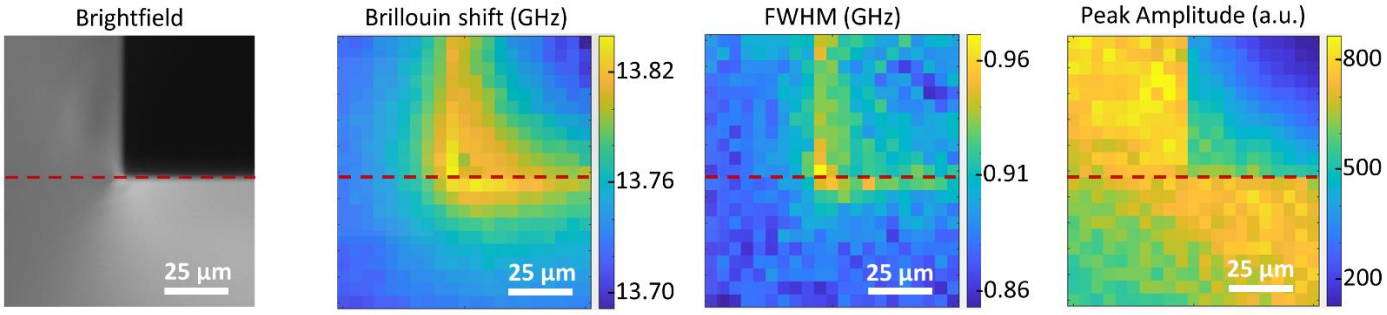

Figure 3: Sharp silica glass corner encapsulated in epoxy glue. The brightfield image is presented on the left, followed by the Brillouin shifts, FWHM, and peak amplitude maps. Red dashed lines have been superimposed to each image along the glass cube boundaries to facilitate image comparison.

ii) Qualitative assessing the peak position precision via non-uniform hydrogel

One of the factors influencing the peak position precision (Figure 1) is the signal-to-noise ratio. The presence of a strong laser line in the vicinity of a Brillouin peak increases the background noise, thus negatively impacting the precision on the peak position. How far a Brillouin peak is located from the laser line as well as our ability to suppress it are thus crucial parameters. The Brillouin peaks of epoxy (~13.7 GHz) are much farther from the laser line when compared to the Brillouin peaks of typical biological samples (usually around 6 GHz). To have a better qualitative representation of the achievable peak position precision, we next looked at non-uniform hydrogels producing Brillouin frequency shifts located around 6 GHz. Figure 4 shows that the total frequency shift variation caused by the density changes across the hydrogel is around 100 MHz. The color map suggests a precision on the Brillouin shift a few MHz. Systematic quantitative experiments and results will be presented in section **v)** below.

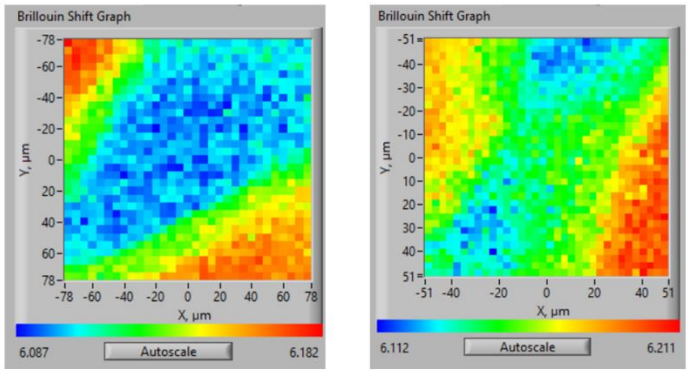

Figure 4: 2D maps presenting the Brillouin frequency shifts of non-uniform hydrogels. The colormap scale is given in GHz.

iii) Onion cell

In order to probe complex samples such as cells, it is not sufficient to achieve sub-picometer spectral resolution; high spatial resolution is also required. Using an objective of 20X, the laser spot size is on the order of 1µm, thus enabling one to resolve small features such as organelles.

Figure 5 presents the Brillouin maps of an onion cell acquisitioned with a 660 nm laser. The Brillouin frequency shift for this cell varied by ~90 MHz (the stiffest region was the nucleus) and the FWHM by ~230 MHz. The precision (standard deviation of repetitive measurements) on the Brillouin frequency shift was ~5 MHz and that of the FWHM was ~10 MHz. Figure 6 shows another example of an onion cell with its nucleus (from a different onion sample). These maps

were obtained using a 532 nm laser and with 25 mW at the sample position. Figure 6 also displays typical spectra from the nucleus (red) and cytoplasm (blue).

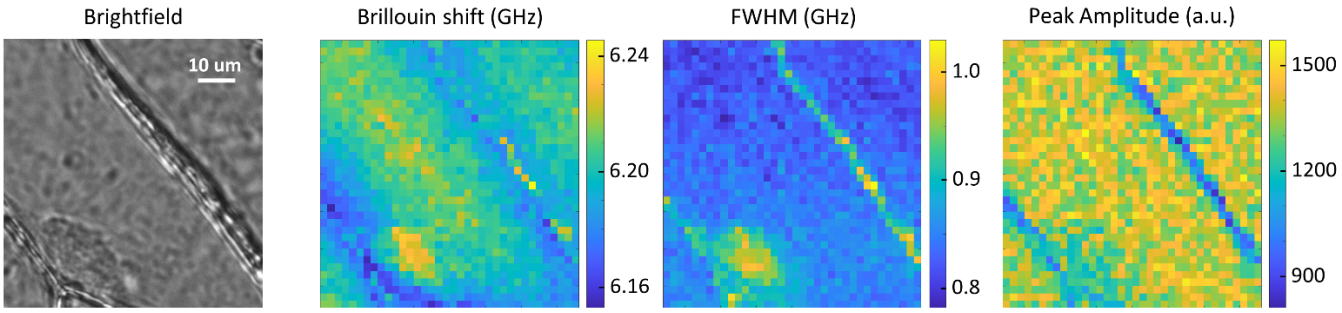

Figure 5: Brightfield image and Brillouin maps (peak shifts, FWHMs, and amplitudes) of an onion cell. The laser power at the sample position was 25 mW (at 660 nm) and the exposure was set to 500 msec per point. The scale bar is 10 μm.

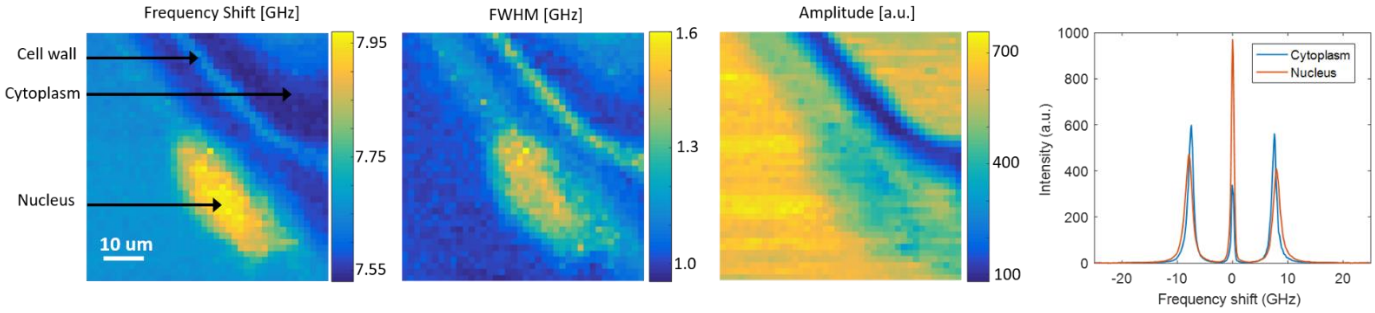

Figure 6: Brillouin maps (peak shifts, FWHMs, and amplitudes) of an onion cell (left) with typical Brillouin spectra for the cytoplasm and nucleus (left). The laser power at the sample position was 25 mW (at 532 nm) and the exposure was set to 200 msec per point. The scale bar is 10 μm.

iv) Mammalian cell spheroid embedded in hydrogel for 3D stack

We can take advantage of the confocal configuration of the excitation/collection system to probe different slices along the Z direction and reconstruct a 3D Brillouin map. Figure 7 below presents the different X-Y maps obtained by probing a sample along five different Z positions with a 660 nm laser.

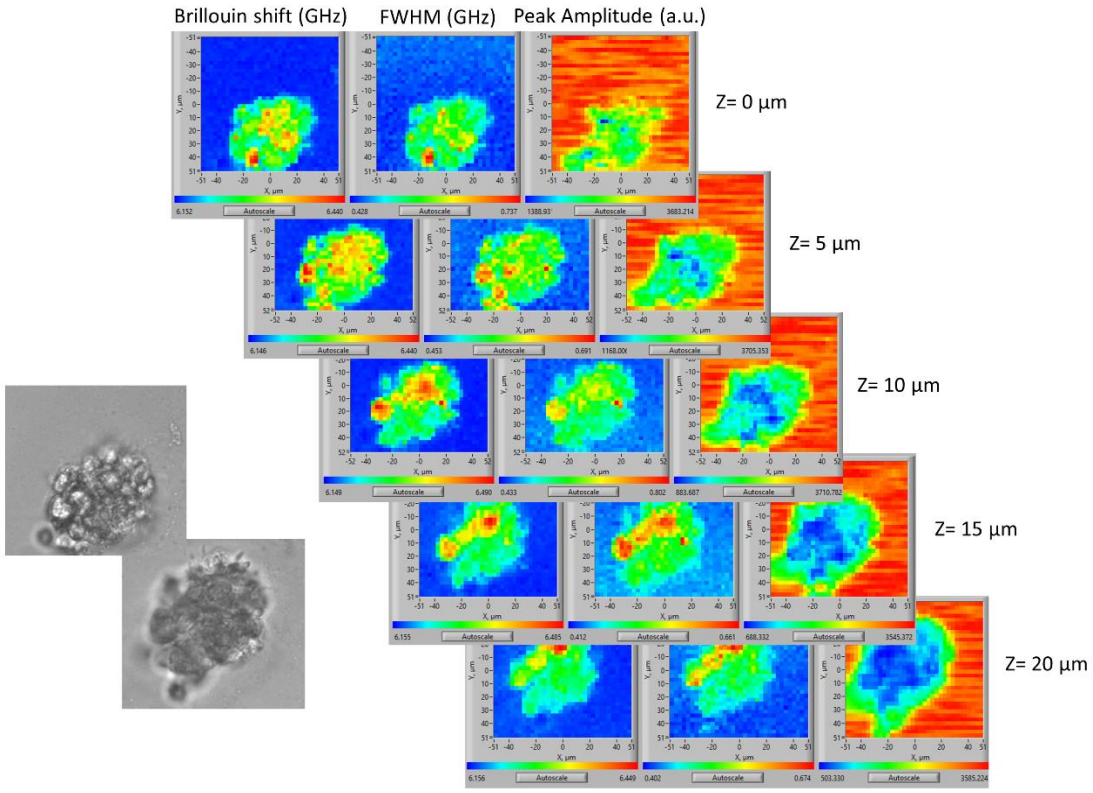

Figure 7: Brillouin maps of a mammalian cell spheroid embedded in hydrogel. 5 slices along the Z direction with step sizes of 5 µm are presented (Z= 0 µm to Z=20 µm). The step sizes along the X and Y directions were set to 3 µm. Each point was acquired with a laser power of ~25 mW (at 660 nm) at the sample position and 300 msec exposure. Courtesy of Prof. Massimo Vassalli, Centre for the Cellular Microenvironment, University of Glasgow.

v) Precision and speed assessment of the system

In the context of acquiring 2D or 3D maps, minimizing the exposure time is paramount. How quickly can a spectrum be acquired with the target signal-to-noise ratio (which in turn will result in the target precision)? As shown in Figure 1, it depends on many factors. Water is a universal (and biologically relevant) reference sample to assess the performances of the system. The results are shown in Figure 8 and Table 1 below. It can be seen that the limit of detection is smaller than 2 msec, using a signal-to-background ratio of 10 as a criterion. More relevantly for mapping, 100 msec of exposure provides a precision better than 3 MHz on the peak position, which is more than required for most applications. Note that those results were obtained using 50 mW of laser power at the sample position; doubling the laser power would half the time required to achieve similar precision.

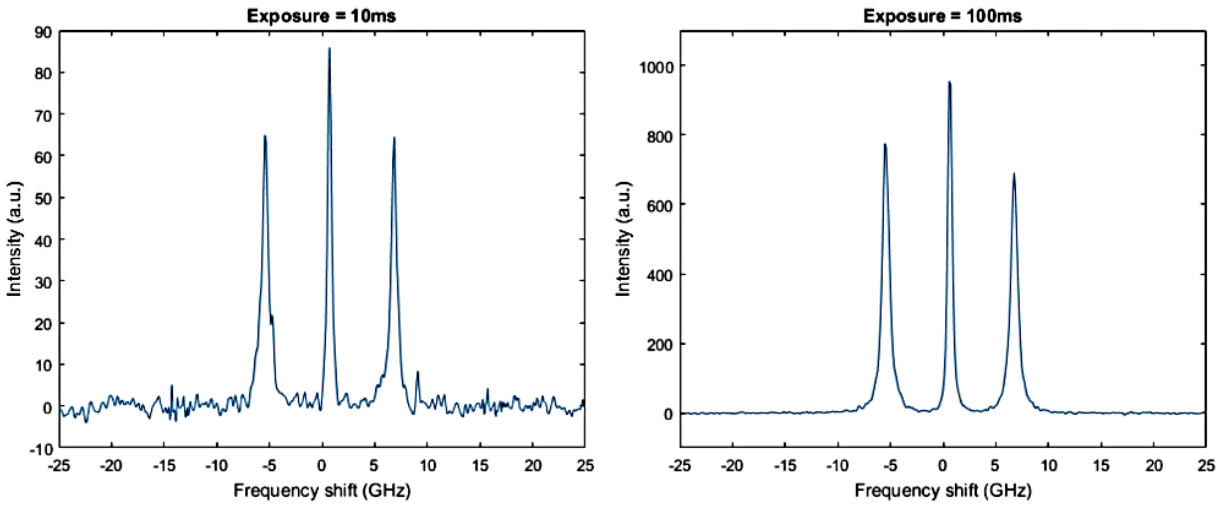

Figure 8: Example of Brillouin signals (from Table 1). For reference only (intensities will vary depending on the config file settings, so SBR measurements are better references). 50 mW of laser power at the sample. 660 nm, 20X objective.

Exposure	Brillouin shift precision	Linewidth precision	Signal-to-background ratio (SBR)
2 ms	62 MHz	93 MHz	11
10 ms	13 MHz	65 MHz	52
100 ms	<3 MHz (2.3 MHz)	10 MHz	506
500 ms	<3 MHz (0.7 MHz)	<10 MHz (6.1 MHz)	$>1 * 10^3$
1000 ms	<3 MHz (1.0 MHz)	<10 MHz (5.5 MHz)	$>1 * 10^3$

Table 1: Precision measurements for reference; analysis of repeated measurements of Brillouin signals in water. [Fit = Lorentzian + constant; SBR = mean(S + AS)/std(+/- 15-25 GHz); for each exposure, 5 repeated measurements were taken.].