Effects of Corneal Hydration on Brillouin Microscopy In Vivo

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PURPOSE. To investigate how corneal hydration affects the Brillouin frequency of corneal stroma.

METHODS. From a simple analytical model considering the volume fraction of water in corneal stroma, we derived the dependence of Brillouin frequency on hydration and hydration-induced corneal thickness variation. The Brillouin frequencies of fresh ex vivo porcine corneas were measured as their hydration was varied in dextran solution and water. Healthy volunteers (8 eyes) were scanned in vivo repeatedly over the course of 9 hours, and the diurnal variations of Brillouin frequency and central corneal thickness (CCT) were measured.

RESULTS. The measured dependence of Brillouin frequency on hydration, both ex vivo and in vivo, agreed well with the theoretical prediction. The Brillouin frequencies of human corneas scanned immediately after waking were on average ~25 MHz lower than their daytime average values. For stabilized corneas, the typical variation of Brillouin frequency was ± 7.2 MHz. With respect to CCT increase or swelling, the Brillouin frequency decreased with a slope of −1.06 MHz/μm in vivo.

CONCLUSIONS. The ex vivo and in vivo data agree with our theoretical model and support that the effect of corneal hydration on Brillouin frequency comes predominantly from the dependence of the tissue compressibility on the water. Corneal hydration correlates negatively with the Brillouin frequency. During daytime activities, the influence of physiological hydration changes in human corneas is < ± 10 MHz. The sensitivity to hydration may potentially be useful in detecting abnormal hydration change in patients with endothelial disorders.

Keywords: cornea biomechanics, Brillouin microscopy, corneal hydration

Brillouin microscopy is an emerging tool originally developed for biomechanical characterization of tissue.[1] It has been applied to evaluate the biomechanical properties of muscle,[2] bone,[3] collagen matrix,[4] and ocular tissue.[5–7] Recently, Brillouin microscopy has received considerable attention for in vivo characterization of the human cornea,[8] particularly for improving the diagnosis of medical conditions in which biomechanical abnormalities occur. While the biomechanical properties, namely longitudinal modulus of tissue, have been the primary target for this technique, this mechanical modulus is related to the speed of sound and known to be sensitive to the water content in tissues[9,10] and water-containing materials.[11] Therefore, besides the composition and ultrastructural organization of the tissue, hydration[12] can also affect the Brillouin optical frequency on which Brillouin microscopy is based.

The dependence of ultrasound speed in the cornea on corneal hydration has been well documented.[13] On the other hand, a strong influence of hydration on corneal stiffness and Young's modulus, which is different from Brillouin-derived longitudinal modulus, has also been measured by traditional mechanical methods such as microindentation[14] and tensile tests.[15] When hydration is changed, corneal thickness varies. The extra fluid enters the stroma tissue, moving collagen fibers in the perpendicular direction to the fiber orientation.[16] The resulting variation of central corneal thickness (CCT) is often used in the clinic to estimate the corneal hydration level of a patient.[12] During normal daytime activities, corneal thickness can vary physiologically, typically <5% during the day[17] but up to 7% in healthy subjects.[18,19] This evidence indicates that the Brillouin frequency of corneal stroma would depend on corneal hydration, and it may even be affected by the natural diurnal changes in corneal hydration. However, to date no studies have been conducted to examine the effect of tissue hydration on Brillouin frequency quantitatively.

Here, we report our investigation into the effect of corneal hydration on Brillouin frequency. We present a theoretical model to derive the dependence of Brillouin frequency on hydration and hydration-induced corneal thickness changes. We performed ex vivo experiments using porcine corneas and verified the model. We then measured the diurnal changes of corneal Brillouin frequency and thickness in vivo in healthy subjects and analyzed the data with respect to the theoretical model. Our results provide quantitative guidelines for the measurement time and interpretation of Brillouin data from patients and offer insights into potential applications based on the hydration sensitivity.

METHODS AND MATERIALS

Brillouin Microscopy Ex Vivo and In Vivo

A custom-built Brillouin imaging system was used for experiments in this study.[7,8] The system uses a 780-nm laser (DL 100, Toptica Photonics AG, Graefelfing, Bavaria, Germany) with an optical power of 5 mW on the sample. The laser beam is focused onto the sample with a microscope objective lens (20×, effective NA = 0.1, Mitutoyo, Kawasaki, Kanagawa, Japan), which provides a lateral resolution of ~5 μm and a ~35-μm axial resolution. Backscattered photons are directed to a Brillouin spectrometer using 2-stage VIPA etalons and an electron-multiplying charge-coupled detector (EMCCD, iX-on897, Andor Technologies, Belfast, Northern Ireland). The spectral resolution of the spectrometer is ~300 MHz. The signal integration time was 0.7 seconds per point for ex vivo samples, and 0.3 seconds per point for human subjects in vivo to minimize artifacts due to patient motion. With an integration time of 0.3 seconds, the shot-noise-limited, standard deviation of the Brillouin frequency measured from water samples was approximately ± 5.2 MHz. For each corneal sample, five axial line scans were acquired in the central region within <2 mm radius from the corneal center. The Brillouin frequencies of all the points in the five scan profiles were averaged to determine the Brillouin frequency of the sample.

Porcine Cornea Samples Ex Vivo in Dextran Solution

We used porcine corneas to investigate the variation of Brillouin frequency due to artificially induced hydration changes. Fresh porcine eyeballs ($n = 3$) were acquired within 12 hours postmortem. The epithelium of all corneas was manually removed with a surgical scalpel, and corneal buttons with ~4 mm diameters were excised from the eyeballs. Immediately after removal, we measured the weight of each corneal button sample (wet tissue weight) using a scientific-grade balance. After the initial measurement, each cornea sample was immersed in 10% dextran solution prepared with distilled water at ~23°C. Every 10 minutes, each sample was taken out of the solution, and the wet tissue weight and Brillouin frequency were measured. During the measurement, which took approximately 3 minutes, we applied drops of dextran solution topically on the sample surface every 1 minute to minimize hydration change of the tissue. After a total immersion time of 40 minutes in the solution, the sample was taken out of solution and placed in the lab environment at ~23°C for 40 minutes. While the sample was drying in the air, we measured the weight and Brillouin frequency every 10 minutes. After the experiment, the corneal sample was left in the air for another 72 hours to dry out completely before we measured the dry weight. The hydration level was determined from H = (wet tissue weight − dry weight)/dry weight.

Porcine Cornea Samples Ex Vivo in Water

We also examined porcine corneal CCT versus Brillouin measurement when corneal hydration is changed by increasing water content in the stroma tissue. Corneal buttons with sclera rings ($n = 3$) were excised from fresh porcine eyeballs obtained within 12 hours postmortem. The samples were sequentially immersed in distilled water to induce artificial swelling. Every 45 minutes (excluding the measurement time of 3 minutes), each sample was removed from the solution and placed in an artificial chamber (Barron Precision Instruments, Grand Blanc, MI, USA) connected to a water column to induce a physiological intraocular pressure (IOP) level of ~15 mm Hg. Then, we measured CCT and central corneal Brillouin frequency. CCT was measured using a custom-built optical coherence tomography (OCT) system[20] by taking cross-sectional images in the central region of the sample. Optical thickness was measured from the images assuming a tissue refractive index of 1.375 at 23°C.[21]

Measurement of Human Corneas In Vivo

To investigate the effect of hydration on Brillouin frequency in vivo, we conducted a pilot study with healthy volunteers in which we measured diurnal changes in corneal central thickness and Brillouin frequency. Four healthy volunteers (male Caucasian, age: 27 ± 1.5 years old) were recruited. The experiment for each subject was performed in 1 day between 8 AM and 6 PM. As soon as the subject woke up, the subject was escorted to the lab with their eyes closed. The first measurement began within 10 to 30 minutes typically, and no later than 1 hour after, the time of waking. We set this first measurement time point to be "0 hour." Measurements of corneal thickness and Brillouin frequency were performed on both eyes. The CCT was measured by an ophthalmologist using a clinical ultrasound pachymeter (SP-100, Tomey Corp., Nagoya, Aichi-ken, Japan).[22] The minimum value from five repeated pachymetry scans was chosen as the CCT. Brillouin frequency was measured from the central corneal region <2 mm from the pupil center. After the first measurement, the subject followed their normal daytime activities, except that they returned to the lab at 1, 2, 3, 6, and 9 hours for CCT and Brillouin measurements. This research followed the tenets of the Declaration of Helsinki, and informed consent was obtained from the subjects after explanation of the nature and possible consequences of the study. This study followed a protocol approved by the institutional review board (IRB) at Massachusetts General Hospital.

Statistical Analysis

The box plot shows the minimum, first quartile, median, third quartile, and maximum. One-way analysis of variance (ANOVA) was conducted to test the effect of time after eye opening on the changes in CCT and Brillouin measurements. Post hoc comparisons were made using the Tukey honest significance difference (HSD) test. Two-tailed Student's *t*-test was used to compare absolute changes in Brillouin frequency due to hydration. Statistical calculations were performed in MATLAB.

RESULTS

Model for the Hydration Dependence of Brillouin Frequency

The Brillouin frequency shift Ω for back scattering Brillouin microscopy is given by[1]:
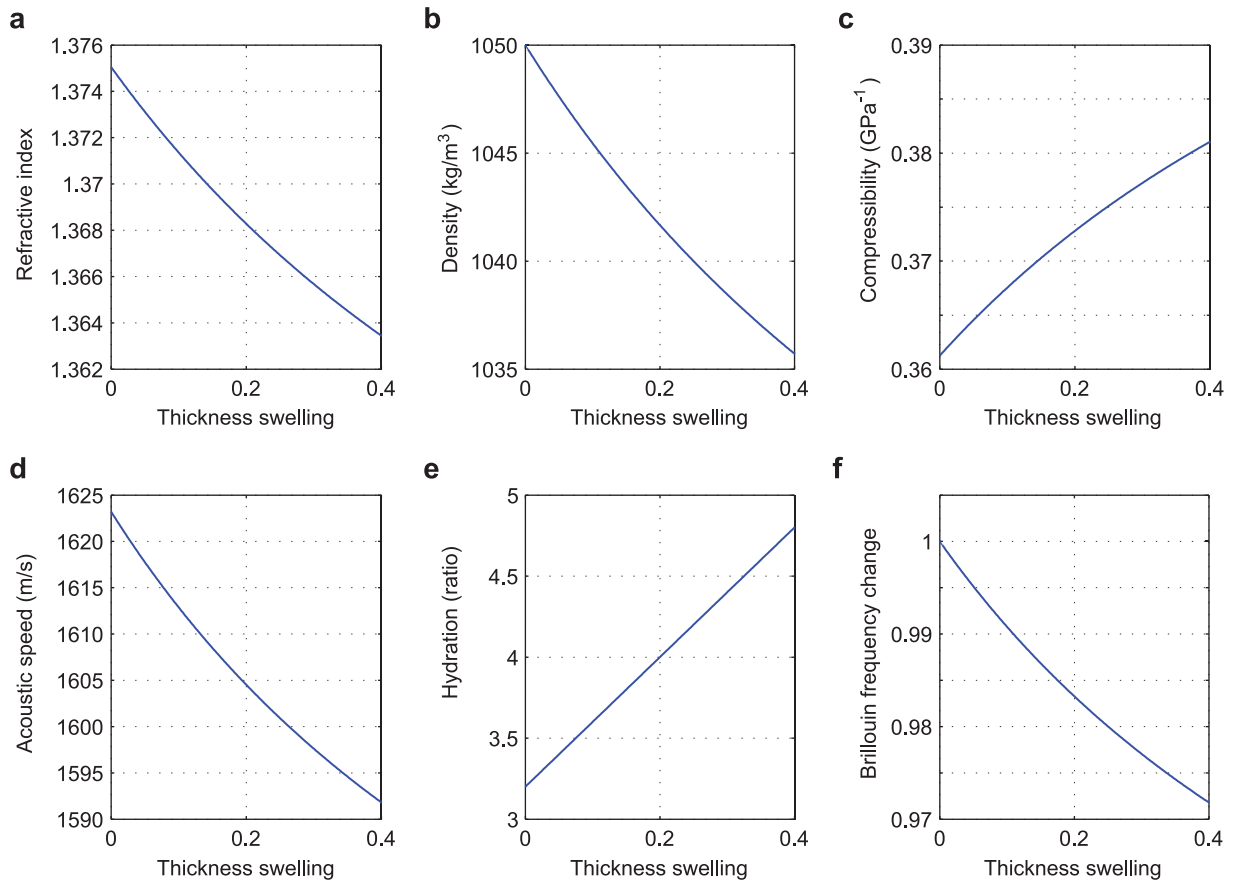
FIGURE 1. Tissue properties of corneal stroma theoretically modeled as a function of normalized thickness change ($\Delta CCT/CCT_0$). (**a**) Refractive index (n). (**b**) Mass density (ρ). (**c**) Compressibility (β). (**d**) Acoustic speed (v). (**e**) Hydration (H). (**f**) Normalized Brillouin frequency change ($\Delta\Omega/\Omega_0$).

$$\Omega = \frac{2}{\lambda} n \sqrt{M/\rho} \approx \frac{2}{\lambda} n \sqrt{1/\rho\beta} \qquad (1)$$

where n, M, ρ, and β are the refractive index, longitudinal modulus, mass density, and compressibility of tissue (corneal stroma), and λ is the optical wavelength. $M = K + 4/3G$, where K and G are bulk and shear moduli, respectively. In soft tissues where $G \ll K$, $M \approx K = 1/\beta$. $\sqrt{1/\rho\beta}$ corresponds to the speed of longitudinal acoustic waves v (at GHz hypersonic frequency).

The hydration level (H) of tissue is defined as:

$$H \equiv \frac{(\text{total tissue weight} - \text{dry weight})}{\text{dry weight}} \qquad (2)$$

This is equivalent to the ratio of the weight of water to dry weight. For most species including humans, water occupies ~76% of corneal stroma by weight. Therefore, the hydration of corneal stroma is close to 3.2 ($= H_0$) in normal physiological conditions.[23] The dry weight comes largely from collagen
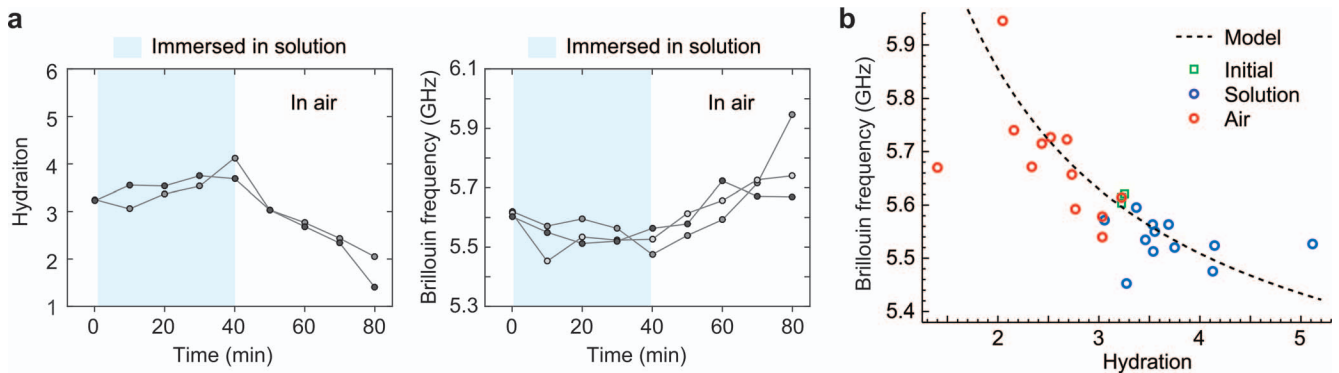
FIGURE 2. Time-lapse variation of the hydration level and Brillouin frequency of porcine corneas ex vivo. (**a**) Measurement data from three samples immersed in 10% dextran solution at $t = 0$ and then removed and exposed to the air at $t = 40$ minutes. (**b**) Comparison of experimental data with the theoretical model.
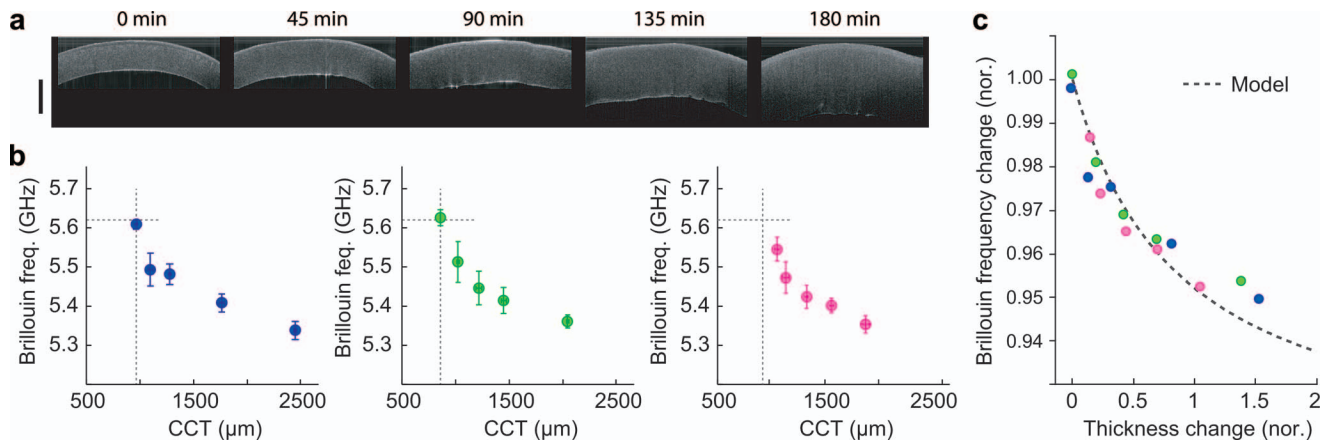
FIGURE 3. Time-lapse measurement of porcine corneas ex vivo immersed in distilled water. (**a**) Five representative OCT images of the central cornea, taken at 45-minute intervals in the experiment. *Scale bar*: 1 mm. (**b**) Measured Brillouin frequency and CCT of three corneal samples. (**c**) Comparison to the theoretical model (*dashed line*). The normalized Brillouin frequency ($\Delta\Omega/\Omega_0$) and swelling ratio were obtained with $\Omega_0 = 5.62$ GHz (23°C) and $\text{CCT}_0 = 0.97$, 0.86, and 0.92 mm, respectively, which are indicated with *dotted lines* in **b**.

fibrils (~15 weight %) and other extrafibrillar matrix components.

To estimate the physical properties in Equation 1, tissues are conveniently modeled to consist of liquid water and "solid" components. The latter are mostly hydrated proteins in a physiological or swollen condition. It is assumed that the properties of these "solid" protein components are independent of hydration and that the liquid water and solid part contribute to the physical properties of the tissue additively. Let V_w denote the volume of water in tissue, and V_s the volume of the solid constituents. Since the total volume is the sum of the two, $V = V_w + V_s$, the volume fraction of water is expressed as $f_w = \frac{V_w}{V_w + V_s}$, and the volume fraction of the solid part is $1 - f_w$. In terms of the volume fraction of water, the refractive index, density, and compressibility[24] can be described:

$$n = n_w f_w + n_s (1 - f_w) \tag{3a}$$

$$\rho = \rho_w f_w + \rho_s (1 - f_w) \tag{3b}$$

$$\beta = \beta_w f_w + \beta_s (1 - f_w) \tag{3c}$$

Here, n_w, ρ_w, and β_w are the refractive index, density, and compressibility of water, and n_s, ρ_s, and β_s are these properties of the solid components. From the definition of hydration, $H = \frac{\rho_w f_w}{\rho_s (1 - f_w)}$. We introduce a variable, x, describing the fractional volume change, defined by

$$f_w = f_{w0}(1 + x); \quad f_{w0} = \frac{\rho_s H_0}{\rho_w + \rho_s H_0} \tag{4}$$

where f_{w0} denotes the volume fraction of water in the normal physiological condition (at $H_0 = 3.2$; the subscript $_0$ indicates the physiological condition). The physiological values of refractive index (n_0) and density (ρ_0) are available in literature. The compressibility values can be determined from the Brillouin frequencies of water (Ω_w) at the body temperature of 37°C and corneal tissues (Ω_0) in vivo. These values are summarized in the Table.

From $f_w = \frac{V - V_s}{V}$, we get $V = \frac{V_s}{1 - f_{w0}(1 + x)}$. Using physiological tissue volume $V_0 = \frac{V_s}{1 - f_{w0}}$, we relate the total volume change to the fractional volume change x via:

$$\frac{\Delta V}{V_0} = \frac{f_{w0} x}{1 - f_{w0} - f_{w0} x} \tag{5}$$

In the cornea, collagen fibrils are arranged parallel to the tissue plane. This specific orientation, as well as the globular
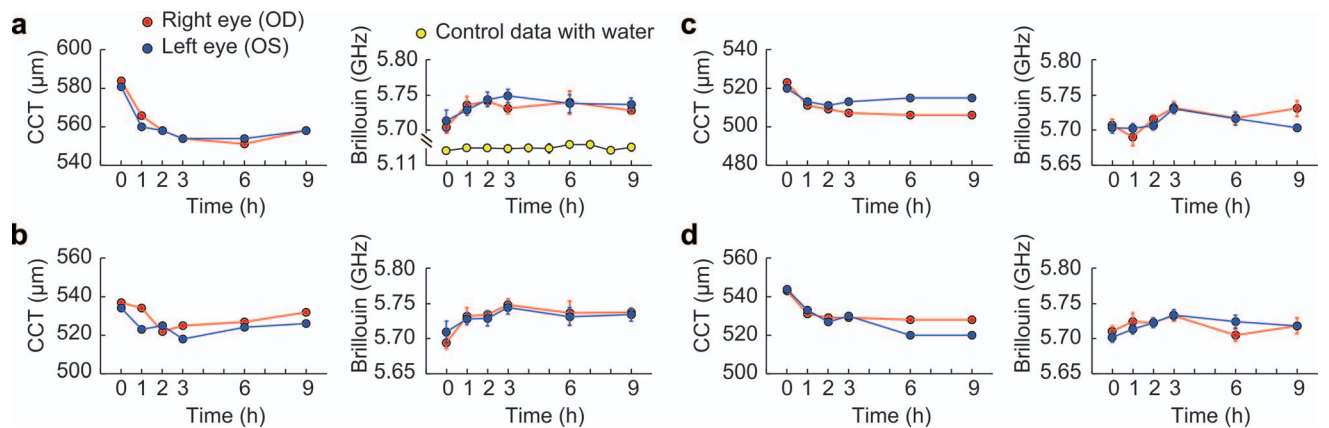
FIGURE 4. In vivo diurnal measurements of the CCT and Brillouin frequency in four healthy human subjects (**a–d**). The first measurements (t = 0) were performed within 10 to 30 minutes of when the subjects first opened their eyes after waking in the morning. Subsequent measurements were made at 1- or 3-hour intervals. Error bars indicate the standard deviation of each data point (±9.3 MHz on average). *Yellow circles* in (**a**) are control data from distilled water at room temperature, showing the stability of the instrument during the daytime.
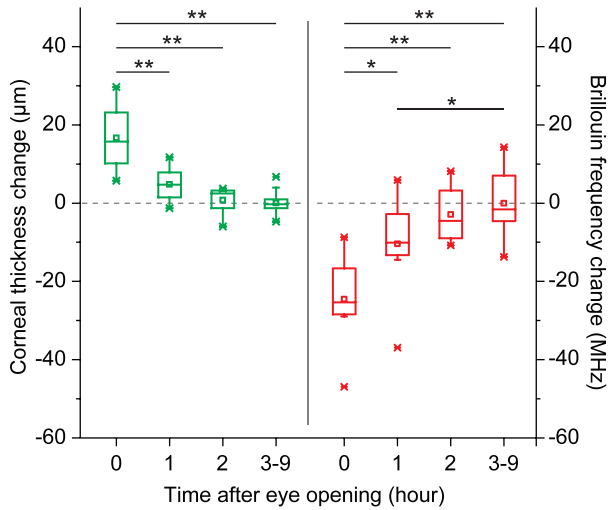
FIGURE 5. CCT change (ΔCCT) and Brillouin frequency change ($\Delta\Omega$) in the hours following eye-opening in the morning. The reference points for these changes are the mean values measured over 3 to 9 hours after waking. *$P < 0.01$, **$P < 0.05$.

geometry of the eye, makes the cornea swell predominantly along the thickness direction and uniformly throughout the entire cornea. For this case of one-dimensional swelling, we can write $\Delta V/V_0 = \Delta\text{CCT}/\text{CCT}_0$, where CCT is the central cornea thickness, CCT_0 is the physiological CCT value (without swelling), and $\Delta\text{CCT} \equiv \text{CCT} - \text{CCT}_0$. Therefore, introducing a variable $l = \Delta\text{CCT}/\text{CCT}_0$, we obtain:

$$x = \frac{(1 - f_{w0})l}{f_{w0}(1 + l)} \quad (6)$$

In Figure 1, we plot the various physical properties of corneal stroma tissue as a function of the normalized thickness change l.
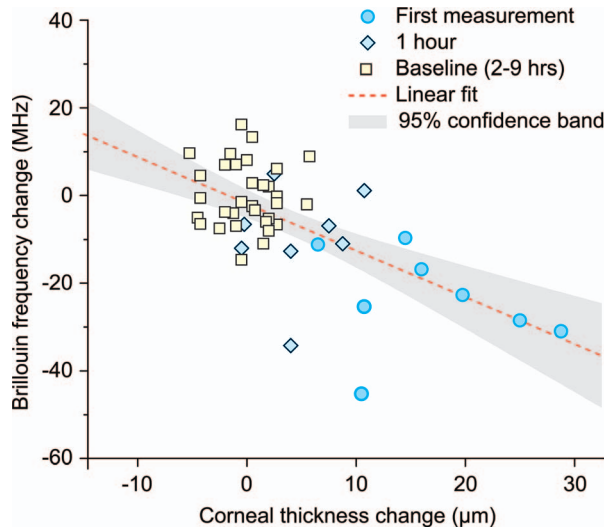
FIGURE 6. Relation between Brillouin frequency change ($\Delta\Omega$) and corneal thickness change (ΔCCT) from eight eyes in vivo (from the data in Fig. 4). *Circles* (*cyan*): early-morning data (t = 0); *diamonds* (*dark gray*): data obtained 1 hour after the first measurement; *squares* (*light gray*): later measurements, after stabilization (2–9 hours); and *dashed line* (*red*): linear curve fit (slope = -1.06 ± 0.2 MHz/μm, Pearson's $r = -0.62$). Baseline is defined as the average value measured over 2 to 9 hours after eye opening.
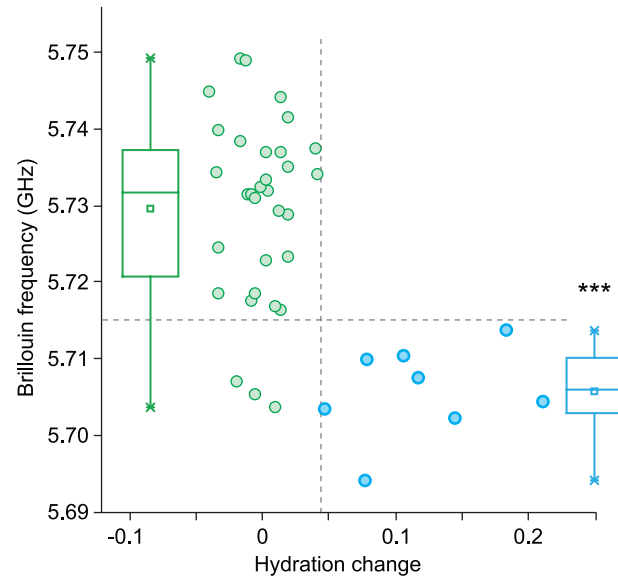
FIGURE 7. Absolute Brillouin frequency values obtained from eight corneas (same as in Figs. 4–6) as a function of hydration change, which was estimated from measured corneal thicknesses using Equation 9. *Cyan circles*: first measurement(s) taken 10 to 30 minutes after waking (t = 0); *Green circles*: data measured over 2 to 9 hours after the first measurement. ***$P < 0.0001$.

In most cases in vivo, the volume and thickness changes are small, that is, $x, l \ll 1$. Using the Taylor expansion, we obtain:

$$\Delta V \equiv V - V_0 \overset{x \ll 1}{\to} V_0 \frac{f_{w0}}{1 - f_{w0}} x \quad (7)$$

and

$$H = \frac{\rho_w f_{w0}(1 + x)}{\rho_s(1 - f_{w0} - f_{w0}x)} \overset{x \ll 1}{\to} H_0\left(1 + \frac{x}{1 - f_{w0}}\right) \quad (8)$$

Using $x \overset{l \ll 1}{\to} \frac{(1 - f_{w0})}{f_{w0}} l$, we derive a linear relationship[12] between hydration and corneal thickness:

$$H - H_0 = a(\text{CCT} - \text{CCT}_0) \quad (9)$$

where $a = \frac{H_0}{f_{w0}}/\text{CCT}_0$. With $H_0 = 3.2, f_{w0} = 0.8$, and $\text{CCT}_0 = 0.54$ mm, we obtain $a = 7.4$ mm^{-1}. This value is in reasonable

TABLE. The Physical Properties of Human Corneal Stroma Used in the Model

Parameters	Human Corneas In Vivo	Reference
H_0	3.2	Ref. 23
f_{w0}	0.80	Equation 4, Ref. 28
n_0	1.375	Ref. 21
n_w	1.335	Ref. 21
n_s	1.531	Equation 3
ρ_0	1050 kg/m^3	Ref. 28
ρ_w	1000 kg/m^3	–
ρ_s	1250 kg/m^3	Equation 3
Ω_0	5.724 GHz	Exp. at $\lambda = 780$ nm
Ω_w	5.219 GHz	at 37°C, Equation 1, Ref. 29
β_0	3.61 10^{-10} Pa^{-1}	Equation 1
β_w	4.31 10^{-10} Pa^{-1}	Equation 1
β_s	0.83 10^{-10} Pa^{-1}	Equation 3
CCT_0	0.54 mm	Ref. 30
Slope	-1.07 MHz/μm	Equation 11

agreement with an experimentally measured value[25] $a = 7.0$ mm^{-1}. As a numerical example, a 1% change in hydration would cause approximately 1% change in corneal thickness ($l = 0.01$; ~5.4 μm) and 0.25% change in water volume fraction ($x = 0.0025$).

For small hydration changes, the relative change of Brillouin frequency can be expressed by taking the derivative on both sides of Equation 1:

$$\frac{\Delta\Omega}{\Omega} = \frac{\Delta n}{n} - 0.5\frac{\Delta\rho}{\rho} - 0.5\frac{\Delta\beta}{\beta} \qquad (10a)$$

$$= \frac{(n_w - n_s)f_{w0}x}{n_0} - 0.5\frac{(\rho_w - \rho_s)f_{w0}x}{\rho_0} - 0.5\frac{(\beta_w - \beta_s)f_{w0}x}{\beta_0} \qquad (10b)$$

Using the values in the Table, we get:

$$\frac{\Delta\Omega}{\Omega_0} = -0.114x + 0.095x - 0.385x \qquad (10c)$$

The contributions from index and density changes (the first and second terms on the right-hand side) approximately cancel each other out.[26] The third term on the right-hand side, the compressibility change, makes the dominant contribution. From $\frac{\Delta\Omega}{\Omega_0} = -0.403x$, we get:

$$\frac{\Delta\Omega}{\Omega_0} = -0.10\frac{\Delta CCT}{CCT_0} \qquad (11)$$

The relative change of Brillouin frequency is a 10th of that of thickness. With $\lambda = 780$ nm, $\Omega_0 = 5.724$ GHz and $CCT_0 = 540$ μm, we calculate a slope of Brillouin frequency with respect to corneal thickness change: $\frac{\Delta\Omega}{\Delta CCT} = -1.07$ [MHz/μm].

Porcine Corneas Ex Vivo

The ex vivo data showed dynamic changes of hydration and Brillouin frequency (Fig. 2a). In the dextran solution, corneal tissues undergo swelling, and we observed a trend of decreasing Brillouin frequency. Once the samples were removed and exposed to the air, the Brillouin frequency increased by approximately 200 MHz within 20 minutes. The plot of experimental data from three different corneas clearly reveals an inverse relationship between Brillouin frequency and hydration (Fig. 2b). To fit the data, we used the model described above, which predicts $\Omega = \Omega_0(1 - 0.114x)$ $(1 - 0.190x)^{-0.5}(1 + 0.770x)^{-0.5}$ and $x = \frac{H-3.2}{4H+3.2}$, where $\Omega_0 = 5.62$ GHz (23°C). The theoretical curve accounts for the experimental data (Fig. 2b). This validates the model. For small hydration changes, using $x \overset{x \ll 1}{\longrightarrow} \frac{(1-f_{w0})(H-H_0)}{H_0}$, we obtain:

$$\frac{\Delta\Omega}{\Omega_0} = -0.082\frac{(H-H_0)}{H_0} \qquad (12)$$

Next, to investigate the relationship between Brillouin frequency and corneal thickness, we performed Brillouin microscopy and OCT measurements of porcine corneal tissues ($n = 3$) as they underwent extensive swelling in distilled water (Fig. 3a). The measured data revealed an inverse relationship between central corneal Brillouin frequency and CCT (Fig. 3b). We converted the measured data to normalized Brillouin frequency change $(\Omega - \Omega_0)/\Omega_0$ with $\Omega_0 = 5.62$ GHz (23°C) for all three samples, and to normalized thickness change $(CCT - CCT_0)/CCT_0$ with $CCT_0 = 0.97$, 0.86, and 0.92 mm, respectively. The plot of these normalized parameters showed an inverse relationship, and the curves of the three corneas collapsed to a universal trend. Our model, $\Delta\Omega/\Omega_0 = (1 - 0.114x)$

$(1 - 0.190x)^{-0.5}(1 + 0.770x)^{-0.5} - 1$ and $x = \frac{\Delta CCT/CCT_0}{4(1+\Delta CCT/CCT_0)}$, accounts for the experimental data well (Fig. 3b).

Human Corneas In Vivo

We observed diurnal variations in both CCT and Brillouin frequency in vivo (Fig. 4). Corneal hydration is known to increase during sleep because of the osmotic influx of water and disabled evaporation through closed eyelids, and is possibly also elevated during a short time window after waking.[18]

We compared the data obtained at different time points after morning eye opening. We grouped the data measured after 3 to 9 hours, expecting a priori that after 3 hours the corneas had sufficient time to stabilize after waking. Figure 5 shows changes in CCT and Brillouin frequency shift with respect to their mean values after stabilization (3–9 hours). The Lilliefors test confirmed normal distribution of the in vivo data at each time point ($P < 0.05$). An ANOVA yielded significant variation of CCT and Brillouin frequency shift with time after eye opening, CCT: F(3,44) = 29.57, $P < 0.001$, Brillouin: F(3,44) = 14.75, $P < 0.001$. Post hoc analyses were conducted using Tukey's HSD test at the α = 0.05 level. The change in CCT measured at 0 hours (17 ± 8 μm) was significantly different from measurements at 1 hour (5 ± 4 μm), 2 hours (1 ± 4 μm), and at 3 to 9 hours (0 ± 3 μm). Similarly, for the Brillouin frequency data, we found significant differences between change at 0 hours (−25 ± 12 MHz) and the changes at 1 hour (−10 ± 13 MHz), 2 hours (−3 ± 7 MHz), and 3 to 9 hours (0 ± 8 MHz). For both the CCT and Brillouin measurements, no significant differences were found between the 2-hour time point and baseline. The changes in Brillouin and CCT measurements in the first 2 hours are very likely due to natural dehydration after waking, as observed in earlier studies.[18]

Based on this finding, we defined the mean CCT and Brillouin frequency measured between 2 and 9 hours after eye opening as the daytime baseline values, called CCT_0 and Ω_0. The scatter plot in Figure 6 shows changes in Brillouin frequency and CCT with respect to baseline for eight corneas. The data indicate a linear relationship between Brillouin frequency and CCT, best fitted with a slope of −1.06 MHz/μm. This coefficient agrees with the prediction of the theoretical model in Equation 11.

Discussion

The experimental data obtained from the ex vivo and in vivo measurements validated the accuracy of our theoretical model. According to the theoretical model and the experiment results, the effect of corneal hydration on Brillouin frequency comes predominantly from the dependence of mechanical compressibility of tissue on the volume fraction of water. An increase in hydration causes an increase in the overall compressibility of the tissue and a decrease in Brillouin frequency.

We did not measure IOP of the subjects for this study on site to avoid any physical perturbation to the cornea by an IOP measurement. All the subjects have healthy corneas and had an eye examination within 12 months before this study, so their IOP levels are expected to be within a normal physiological range. The subjects may have some diurnal variation of IOP. However, we believe this effect on the Brillouin frequency would be much smaller than the measured effect of diurnal hydration changes. Our future work will investigate the relationship between IOP and Brillouin frequency.

The shot-noise-limited, standard deviation error of the clinical Brillouin microscope used in this study was approxi-

mately ± 5.2 MHz at 0.3-second integration time.[27] From Equations 11 and 12, the noise-equivalent sensitivity to corneal hydration is $\pm 1.1\%$ ($\Delta H/H_0$), and the noise-equivalent sensitivity to CCT change is ± 4.7 µm. This sensitivity has implications for the potential clinical applications of Brillouin microscopy.

First, to determine the normal Brillouin frequency of a patient, measurement should be conducted at least 2 hours after waking. The mean-subtracted Brillouin data ($\Delta\Omega$) during t = 2–9 hours had a standard deviation of 7.2 MHz. This small deviation indicates that corneal hydration is stable enough (once the initial dehydration upon waking has occurred) that the daytime variation in Brillouin frequency may be almost negligibly small.

Second, hydration can be a compounding factor in interpreting the Brillouin frequency solely from a biomechanical standpoint. The physiological corneal hydration level during the daytime hours (after morning stabilization) can vary slightly among individuals, but the interpersonal difference in hydration is hard to measure independently. The mechanical compressibility of corneal stroma, which determines the Brillouin frequency, is also affected by changes in the compressibility β_s of the solid components (collagen fibrils and other structural proteins). Considering this contribution in Equation 3c, we get $\Delta\beta = (\beta_w - \beta_s)\Delta f_w + \Delta\beta_s(1 - f_{w0})$. Putting this into Equation 10 and using $\Delta x = (1 - f_{w0})\Delta H/H_0$, we obtain

$$\frac{\Delta\Omega}{\Omega_0} \approx -0.08\frac{\Delta H}{H_0} - 0.1\frac{\Delta\beta_s}{\beta_0} \qquad (13)$$

The first term on the right-hand side corresponds to the contribution of hydration change, and the second term describes the mechanical variation of the stromal solid part. From the instrument uncertainty of 5.2 MHz, the noise-equivalent sensitivity to the solid-part compressibility is $\Delta\beta_s/\beta_s = 4\%$.

We examined the absolute Brillouin frequency values in the data set (without subtracting the baseline values) with respect to hydration (Fig. 7). The daytime variation of the absolute Brillouin frequency was 12 MHz, modestly but statistically significantly larger than the standard deviation of 9.3 MHz of each data point (in Fig. 4). In our recent clinical study, the Brillouin values measured from 47 healthy subjects over a wider age range (age: 39 ± 13 years old, 24 females, 23 males) were between 5.69 and 5.76 GHz with a standard deviation of 15 MHz.[27] If we assume that the person-to-person variability of normal hydration levels in healthy population is negligible ($\frac{\Delta H}{H_0}$ < 1%), the measured interpersonal variability could be attributed to differences among individuals in the mechanical compressibility of the solid stromal part, and therefore, it has a biomechanical origin.

Third, patients with endothelial disorders can develop considerably different corneal hydration levels compared to the healthy population. For example, Fuchs' corneal dystrophy is associated with the loss of endothelial cell function, resulting in elevated hydration and corneal swelling. Brillouin microscopy has the potential to detect abnormal hydration changes in Fuchs' corneas. In the plot of absolute Brillouin frequency values (Fig. 7), 100% of the data points measured at t = 0 were lower than 5.715 GHz, and 29 of 32 (90%) data points obtained in the daytime (t = 2–9 hours) were above the cutoff line (horizontal dashed line). Central Brillouin frequency outside the normal range (for example, <5.70 GHz) may be an indicator of corneal diseases involving endothelial function, as well as keratoconus and other biomechanical disorders.[27] Further clinical studies are warranted to explore this diagnostic potential.

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