Selective two-photon collagen crosslinking *in situ* measured by Brillouin microscopy: supplementary material

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This document provides supplementary information to "Selective two-photon collagen crosslinking measured by Brillouin microscopy", <http://dx.doi.org/10.1364/optica.3.000469>. The first section contains detailed information on the experimental methods, including sample preparation, crosslinking protocol, Brillouin measurements, and post-processing. The second section contains theoretical considerations, including comparison of laser parameters for one-photon and two-photon crosslinking, and calculation of the longitudinal modulus. The last section contains the supplementary figures cited in the manuscript. © 2016 Optical Society of America

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1. MATERIALS & METHODS:

A. Corneal Preparation and Staining:

Fresh young bovine (<10 days) eyes were acquired and temporarily stored in ice-cold mineral oil to maintain hydration. After excess fat and optic nerve were removed from the eyes, the corneas were excised and stained with riboflavin according to the Dresden Protocol (epi-off), with the modification that the working riboflavin solution was 10% dextran w/v%, rather than 20%. The reason for this was that our preliminary results (data not shown) had determined that a 20% dextran riboflavin solution caused significant dehydration of the cornea, which introduced z-drift during the subsequent crosslinking and imaging steps. A 10% dextran solution prevented these unwanted effects. Note that the corneas were kept at 4° C during all steps of the above described procedure. Following staining, a central region of the cornea was removed using a 10 mm biopsy punch, mounted on a glass-slide, and gently compressed with a No. 1.5 coverslip for two-photon imaging and crosslinking.

B. Corneal Crosslinking:

For imaging and crosslinking, we used a home-built, video-rate, two-photon microscope. The system equipped a mode-locked

Ti:Sapphire laser (MaiTai DeepSee eHP, Newport) as a light source which provides ~ 150 fs pulses at 80 MHz repetition rate. The scanning unit is composed of a polygon mirror for fast axis (x) and a galvanometer for slow axis (y), allowing frame rate of 30 Hz. Using a 20x, NA 0.9 water-immersion objective, the scanning area has dimensions of (290 x 480) μm^2 . The emitted light was detected by photomultiplier tubes (PMT) through appropriate dichroic and bandpass filters. One PMT was used for detection of second harmonic generation (400 nm $\pm$ 5 nm) and another for riboflavin fluorescence (520 nm $\pm$ 35 nm).

Before imaging, the corneas were allowed to warm to room temperature to minimize temperature induced drift effects. Further XY-drift correction was implemented during post-processing in MATLAB. The top of the corneal stroma was determined by determining the Z-plane where SHG intensity abruptly changed. All depths are given relative to the top of the corneal stroma. To ensure that riboflavin had completely penetrated the cornea, a full Z-stack of a small XY-section of the cornea was first taken. 2P-CXL was performed at an average power of 104 mW, for 10 minutes per plane. To demonstrate three-dimensional, subsurface 2P-CXL, we crosslinked a vertically-aligned stack of 10 planes, equidistantly spaced 3 μm apart at depths of approximately -35 to -65 μm . The

crosslinking was monitored in real time and the riboflavin fluorescence and SHG intensity were recorded at 5 second intervals. If non-negligible drift was observed during this process, crosslinking was restarted with a different sample.

C. Brillouin Imaging:

The confocal Brillouin microscope is similar to previously described [1,2]. A single-mode, 532 nm, frequency-doubled Nd-YAG laser (Torus, Laser Quantum, Inc.) was used to illuminate the same with typical powers of 7 mW. A 10x, 0.3 NA air objective lens was used in the inverted, epi-illumination configuration. The scattered light was collected by a single-mode optical fiber, which also confers confocality, and delivers light to the high-resolution, apodized, two-stage VIPA spectrometer. Linearly variable intensity filters (Rugate and Newport 50FS04DV) were used for apodization. The diffracted light following the two-stage VIPA was detected with an EM-CCD camera with a dispersion slope of 0.3 GHz/pixel. The exposure time per pixel was 0.3 s. The image acquisition time varied from 0.5-2 hours, depending on the number of pixels. Calibration with reference materials was performed before each measurement as previously described.

Following crosslinking of a corneal sample, the XY location of the crosslinked region was marked on the glass slide with a marker and corneas were transferred to the Brillouin microscope. To minimize dehydration during imaging the corneas were submerged in mineral oil, and placed upside down on a glass-bottom dish for illumination from the bottom. The sample was translated in three-dimensions using a motorized stage (Prior Scientific, Inc.) with steps-sizes of 2-25 μm depending on the resolution required. The crosslinked region, as well as the surrounding areas, was thoroughly imaged by taking XY and XZ slices through the cornea.

D. Post-Processing:

To remove the PMT and laser-intensity noise from the collected two-photon microscope images, a combination of a median filter and a notch filter, respectively, were applied using custom-written MATLAB software. The filtered images were then analyzed for mean SHG, mean riboflavin intensity, and collagen fiber orientation. The fiber orientation was determined via a previously described protocol [3]. Briefly, edge detection and filtering were used to generate a binary mask of the collagen fibers from each SHG image. The inverse of this binary mask was multiplied with the original filtered image to suppress the non-collagen signal (Fig. S1). The FFT of the resultant image was taken and a bimodal Von Mises distribution was fit to the power in each radial angle of the output FFT. The mean of this distribution gave the average collagen fiber orientation in the image and the variance specified the alignment of the fibers.

The collected Brillouin data also required further processing. For each imaged voxel, the Brillouin microscope gives a spectral pattern consisting of the scattered light intensity as a function of Brillouin shift. This spectrum can be described by a Lorentz distribution with a mean equal to the characteristic Brillouin shift of the material. To determine the characteristic Brillouin shift of each voxel we used a least-squares error minimization methodology to fit a Lorentzian distribution to its output. A preliminary heat map of Brillouin shifts for each 2D Brillouin XY-slice was generated, and a 2D-median filter was applied to minimize noise. Using the corresponding Z location of each XY-slice, we then applied a 3D linear interpolation algorithm to generate a preliminary 3D view of the imaged Brillouin region. We then applied a 3x3x3 box kernel to generate the final 3D image. The relevant code was implemented in MATLAB.

2. THEORY

A. Comparison of laser parameters used for 1P and 2P-CXL
Here, we estimate the fluence and exposure time needed for 2P-CXL to match the laser parameters used for 1P-CXL with riboflavin and UVA light.

The number of excitations per molecule during the course of conventional one-photon CXL is expressed as follows:

$$N_{1p} = \frac{P * \sigma_{1p} * t}{A * h\nu_1} = 360 \frac{\text{excitations}}{\text{riboflavin molecule}} \quad (\text{S1})$$

Here, A is the illuminated area, Power/Area (P/A) gives fluence, which is typically 3 mJ/(s*cm²) at 365 nm, σ_{1p} is the 1P absorption cross-section of riboflavin which is $3.6 \times 10^{-17} \text{cm}^2$, t is 30 min, and $h\nu_1$ is the photon energy at 365 nm.

In contrast, the two-photon excitation rate depends on the spatial and temporal profile of the laser. We can express the total number of molecular excitations by integrating the intensity function squared over time and space, as follows:

$$N_{2p} = \frac{1}{2} \frac{\sigma_{2p}}{(h\nu_2)^2} * t f l C \iiint_{-\infty}^{\infty} I^2(x, y, t) dx dy dt \quad (\text{S2})$$

where the 1/2 factor accounts for the two photons needed for two-photon absorption, tf represents the number of laser pulses within given time t , l is the optical path length, and C is the riboflavin concentration in molecules per unit volume.

Then the number of excitations per molecule is:

$$N_{2p} = \frac{1}{2} \frac{\sigma_{2p}}{(h\nu_2)^2} * \frac{t f l}{V} \iiint_{-\infty}^{\infty} I^2(x, y, t) dx dy dt \quad (\text{S3})$$

where V is the excitation volume.

We can approximate the excitation volume divided by the optical path length to be equal to the 2P excitation area is approximated by the area of an ellipse: $A_{2p} = \pi \Delta x \Delta y$:

$$N_{2p} = \frac{1}{2} \frac{\sigma_{2p}}{(h\nu_2)^2} * \frac{t f}{\pi \Delta x \Delta y} \iiint I^2(x, y, t) dx dy dt \quad (\text{S4})$$

Given Gaussian profiles for $I(x, y, t)$:

$$I(x, y, t) = I_0 * e^{-t^2/\tau^2} * e^{-x^2/(\Delta x)^2} * e^{-y^2/(\Delta y)^2} \quad (\text{S5})$$

Squaring and integrating over $x = -\Delta x$, $y = -\Delta y$ to Δy , and $t = -\Delta t$ to Δt , which correspond to the $1/e^2$ widths of the spatial and temporal profiles:

$$\iiint I^2(x, y, t) dx dy dt = (I_0)^2 * \left(\frac{\pi}{2}\right)^3 * \tau \Delta x \Delta y * c \quad (\text{S6})$$

where c is a scaling factor equal to $\left[\frac{\int_{-\infty}^{\infty} e^{-2r^2} dr}{\int_{-\infty}^{\infty} e^{-r^2} dr}\right]^3 \cong 0.87$

Equation S6 is in terms of peak irradiance (I_0). This can be expressed by an average power P_{avg} by the relation:

$$P_{avg} = f \iiint_{-\infty}^{\infty} I(x, y, t) dx dy dt = \pi^2 I_0^2 f \tau \Delta x \Delta y \quad (\text{S7})$$

where f is the repetition rate (Hz). Thus, we have:

$$(I_0)^2 = \frac{P_{avg}^2}{\tau^2 f^2} * \frac{1}{\pi^3} * \frac{1}{(\Delta x \Delta y)^2} \quad (\text{S8})$$

Combining Equations (S2-S8), we obtain:

$$N_{2p} \cong \frac{c}{4(2\pi)^{1/2}} * \frac{\sigma_{2p}}{(h\nu_2)^2} \frac{t}{\tau f} P_{avg}^2 \frac{1}{(A_{2p})^2} \quad (S9)$$

Here, σ_{2p} is 0.306 GM at 810 nm, τ is 150 fs, f is 80 MHz, while A_{2p} is calculated based on fitting of the illumination point spread function squared (IPSF²) to a Gaussian function. The $1/e^2$ radius (r_{2p}) of the IPSF² is expressed as [4]:

$$r_{2p} = \frac{0.325 \lambda}{NA^{0.91}}, \text{ for } NA > 0.7 \quad (S10)$$

where NA is the numerical aperture and λ is the wavelength of the incident radiation. For NA=0.9, we have $r=0.29 \mu\text{m}$, and $A_{2p} = \pi r^2 \cong 2.18 \times 10^{-9} \text{ cm}^2$.

Given an appropriate, non-toxic power (100 mW at 810 nm), and assuming $N = N_{2p} = N_{1p}$ we can solve for the exposure time needed:

$$t = \frac{4(2\pi)^{1/2}}{c} \frac{N(h\nu_2)^2 \tau f}{\sigma_{2p} P_{avg}^2} (A_{2p})^2 = 0.46 \text{ ms} \quad (S11)$$

The required fluence (F) for 2P-CXL is:

$$F = 0.46 \text{ ms} * \frac{100 \text{ mW}}{2.2 \times 10^{-9} \text{ cm}^2} = 2.1 \times 10^{-4} \text{ J/cm}^2 \quad (S12)$$

Since 2P-CXL requires a focused beam which is scanned over a larger area, the illumination duty cycle must be taken into account to compute the practical exposure time, t' . For our system, A_{2p} is dispersed over a $(290 \times 480) \mu\text{m}^2$ scanning area:

$$t' = t * \frac{\text{Scanning area}}{A_{2p}} = 4.9 \text{ min} \quad (S13)$$

B. Calculation of longitudinal modulus from Brillouin shift

Spontaneous Brillouin scattering occurs through photon-phonon interactions inside the material or tissue. The Brillouin shift (Ω) is given by [5]:

$$\Omega = 2K \sin\left(\frac{\theta}{2}\right) \sqrt{\frac{M'}{\rho}} \quad (S14)$$

where K is the photon wavenumber, θ is the angle between incident and scattered photons, M' is the real part of the longitudinal modulus, and ρ is the mass density. We can see that the Brillouin shift is proportional to the propagation velocity $\sqrt{M'/\rho}$ of GHz acoustic waves inside the material.

For our epi-detection scheme, $\theta = \pi$, such that M' can be rewritten as:

$$M' = \frac{\rho \lambda^2 \Omega^2}{4n^2} \quad (S15)$$

where λ is the wavelength of the incident light, and n is the refractive index. ρ/n^2 can be assumed to be approximately constant throughout the cornea at 0.56 g/cm^3 .

2. SUPPLEMENTARY FIGURES

Supplementary Figure S1. (a) Raw SHG image of collagen fibers 50 μm below the cornea surface. (b) SHG image after median filtering of laser and PMT noise. (c) Image after edge detection and suppression of non-collagen signal. (d) 2D-FFT magnitude spectrum of c, which was used to quantify collagen fiber orientations. Scalebar: 50 μm .

Supplementary Figure S2. Representative SHG images of bovine cornea pre and post application of riboflavin/dextran solution, as well as post 1P-CXL. Images shown are approximately 100 μm below the cornea surface. Scale bar: 50 μm

Supplementary Figure S3. Depth profile of riboflavin fluorescence prior to crosslinking. Red line is corrected for attenuation of collected light at 530 nm, assuming an attenuation coefficient of 10 cm^{-1}

Supplementary Figure S4. (a) x-y image of 2P-CXL treated cornea, at 40 μm below the cornea surface. Step size: $x=25\text{ }\mu\text{m}$, $y=10\text{ }\mu\text{m}$. (b) x-z image of 2P-CXL treated cornea. Step size: $x=5\text{ }\mu\text{m}$, $z=4\text{ }\mu\text{m}$.

Visualization 1 (Movie File). Three-dimensional view of the 2P-CXL region reconstructed and interpolated from thirteen x-y images at 10 μm intervals in z . See Fig. 5.

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