SUPPLEMENTARY INFORMATION

Theory and procedures of image processing

1. Background subtraction

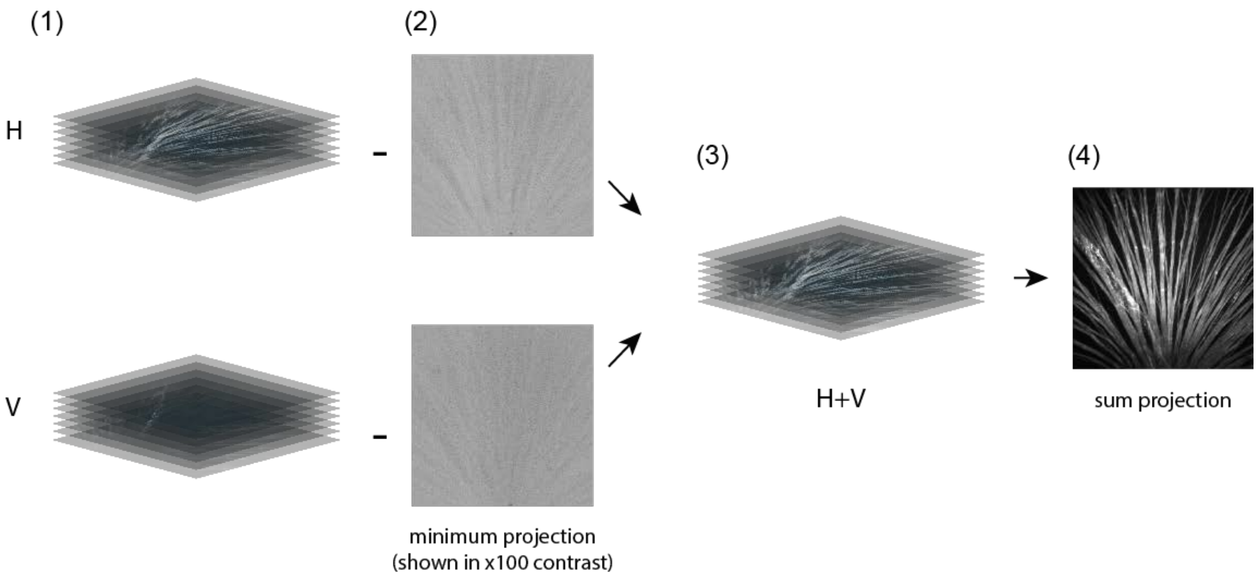

Figure S1. Background subtraction and combination of two input polarizations

1) Take a minimum projection of z-stack images for two orthogonal linear polarizations of excitation, i.e., horizontal (H) and vertical (V).
2) Subtract the minimum projection from each z-sections, eliminating background and fixed pattern noises.
3) Combine background-subtracted z-sections to obtain z-stack images of total SHG intensity.
4) Take a sum projection to obtain a 2D map of SHG intensity.

2. Geometrical correction of the integrated SHG intensity

Fig. S2 illustrates the geometry of MT in terms of the polar and azimuthal angles θ and φ relative to the excitation beam. The Cartesian coordinates are (X, Y, Z) and (x, y, z) in the frames of reference relative to the beam and the molecule, respectively.

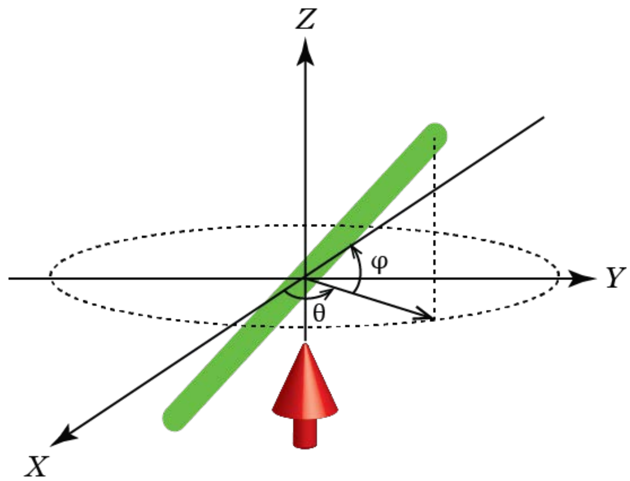

Figure S2. The orientation of MT (green) relative to the excitation beam (red).

The excitation beam linearly polarized along the X-axis has the components of electric field along the molecular coordinates.

$$E_x = E_0 \cos\theta \cos\varphi \quad \text{(S1)}$$

$$E_y = E_0 \sin\theta \quad \text{(S2)}$$

$$E_z = E_0 \cos\theta \sin\varphi \quad \text{(S3)}$$

The SHG polarization is given by

$$P_x = \chi_{xxx}E_xE_x + \chi_{xyy}E_yE_y + \chi_{xzz}E_zE_z + 2\chi_{xxy}E_xE_y + 2\chi_{xyz}E_yE_z + 2\chi_{xzx}E_zE_x \quad \text{(S4)}$$

$$P_y = \chi_{yxx}E_xE_x + \chi_{yyy}E_yE_y + \chi_{yzz}E_zE_z + 2\chi_{yxy}E_xE_y + 2\chi_{yyz}E_yE_z + 2\chi_{yzx}E_zE_x \quad \text{(S5)}$$

$$P_z = \chi_{zxx}E_xE_x + \chi_{zyy}E_yE_y + \chi_{zzz}E_zE_z + 2\chi_{zxy}E_xE_y + 2\chi_{zyz}E_yE_z + 2\chi_{zzx}E_zE_x \quad \text{(S6)}$$

Assuming that MT is a uniaxial molecule with C_∞ symmetry and also that the Kleinmann symmetry applies to allow permutation of indices, the second-order susceptibility has only two independent, non-vanishing elements, i.e., χ_{xxx} and $\chi_{xyy} = \chi_{yxy} = \chi_{yyx} = \chi_{xzz} = \chi_{zxz} = \chi_{zzx}$. As a result,

$$
\begin{aligned}
P_x &= \chi_{xxx}E_xE_x + \chi_{xyy}E_yE_y + \chi_{xzz}E_zE_z \\
&= E_0^2\left(\chi_{xxx}\cos^2\theta\cos^2\varphi + \chi_{xyy}\sin^2\theta + \chi_{xzz}\cos^2\theta\sin^2\varphi\right)
\end{aligned} \tag{S7}
$$

$$P_y = 2\chi_{yxy}E_xE_y = E_0^2 \cdot \chi_{yxy}\sin 2\theta\cos\varphi \tag{S8}$$

$$P_z = 2\chi_{zzx}E_zE_x = E_0^2 \cdot \chi_{zxz}\cos^2\theta\sin 2\varphi \tag{S9}$$

The SHG intensity is

$$I_{SHG,X} = P_x^2 + P_y^2 + P_z^2 \tag{S10}$$

and the total SHG intensity, which is the sum of the SHG signals for two linear polarizations along the X- and Y-axis, respectively, is

$$
\begin{aligned}
I_{SHG}(\theta,\varphi) &= I_{SHG,X} + I_{SHG,Y} \\
&= E_0^4\Big\{\left(\chi_{xxx}\cos^2\theta\cos^2\varphi + \chi_{xyy}\sin^2\theta + \chi_{xzz}\cos^2\theta\sin^2\varphi\right)^2 \\
&+ \left(\chi_{yxy}\sin 2\theta\cos\varphi\right)^2 + \left(\chi_{zxz}\cos^2\theta\sin 2\varphi\right)^2 \\
&+ \left(\chi_{xxx}\sin^2\theta\cos^2\varphi + \chi_{xyy}\cos^2\theta + \chi_{xzz}\sin^2\theta\sin^2\varphi\right)^2 \\
&+ \left(\chi_{yxy}\sin 2\theta\cos\varphi\right)^2 + \left(\chi_{zxz}\sin^2\theta\sin 2\varphi\right)^2\Big\}
\end{aligned} \tag{S11}
$$

In terms of the ratio γ between the on- and off-diagonal elements,

$$
\begin{aligned}
I_{SHG}(\theta,\varphi) = I_0\{&8(\gamma-3)(\gamma+1)\cos^4\varphi\cos 4\theta + 3(\gamma-3)(\gamma+1)\cos 4\varphi \\
&+ 4(3\gamma-1)(\gamma+1)\cos 2\varphi + (9\gamma^2+14\gamma+69)\}, \qquad \gamma = \chi_{xxx}/\chi_{xyy}
\end{aligned} \tag{S12}
$$

The value of γ is 3 for MT[1], in which case the SHG intensity is independent of the polar angle .

$$I_{SHG}(\varphi) = I_{SHG}(0)(0.4\cos 2\varphi + 0.6) \tag{S13}$$

The azimuthal, or tilt, angle was calculated at every pixel from the topography of the retina $\Delta z(x, y)$, which was obtained from the z-coordinate of the automated stage and the axial positions of the top surface of the retinal nerve fibers. Then the integrated SHG intensity is

$$I_{SHG}(\varphi) = I_{SHG}(0)\left(0.8/\left(1 + \left(\frac{dz}{dr}\right)^2\right) + 0.2\right) = I_{SHG}(0)/C \qquad \text{(S14)}$$

$$\therefore I_{SHG}(0) = I_{SHG}(\varphi) \cdot C \qquad \text{(S15)}$$

3. Tilt correction

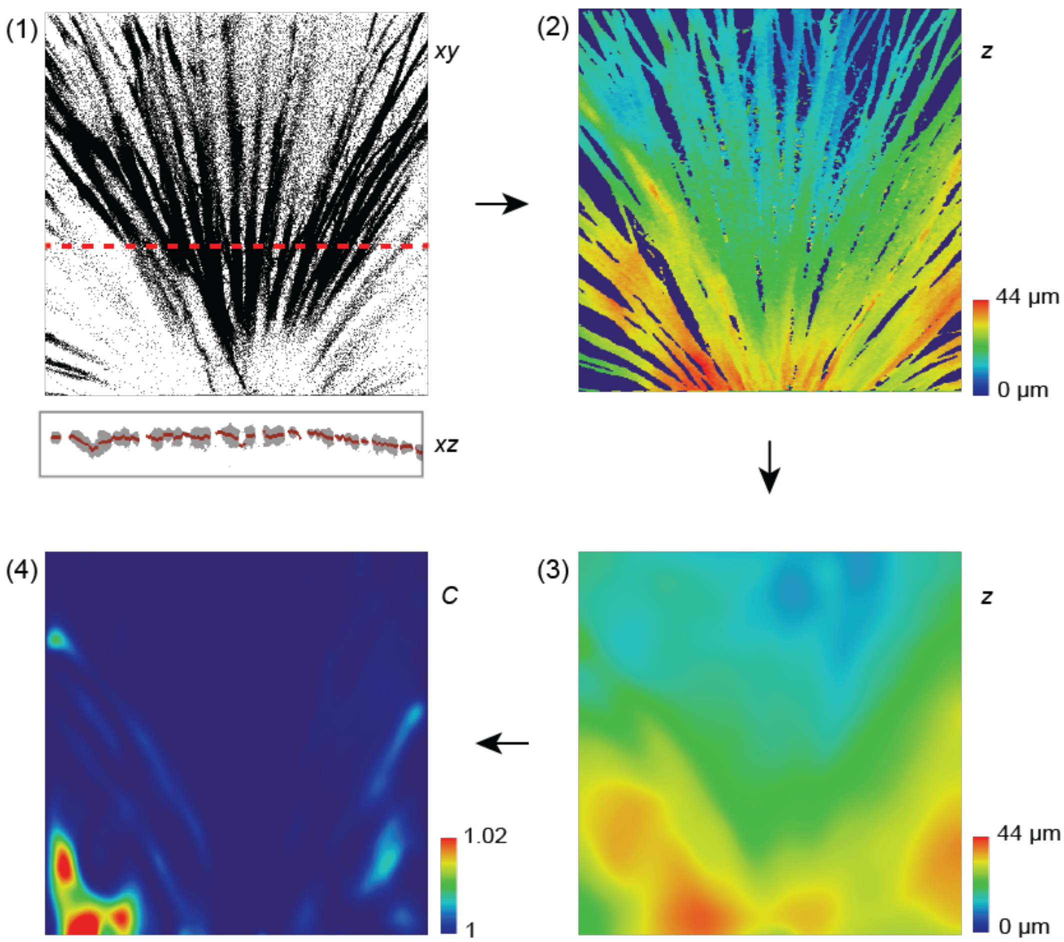

Figure S3. Tilt correction

1) Resample the thresholded z-stack (xy) into y-stacks (xz), and then find the center of the cross section of the nerve across the retina to obtain the height map (z).

2) Interpolate, filling the non-nerve gaps and eliminating borders, to obtain the topography of the retinal flatmount. Apply median filtering to eliminate speckles and remove high spatial frequencies.
3) Take radial gradients $\frac{dz}{dr}$ and evaluate the correction factors (C). The magnitude of errors due to tissue topography was typically <~1%.
4) C is then multiplied with the image of SHG intensity, according to Eq. S15.

4. Morphometry

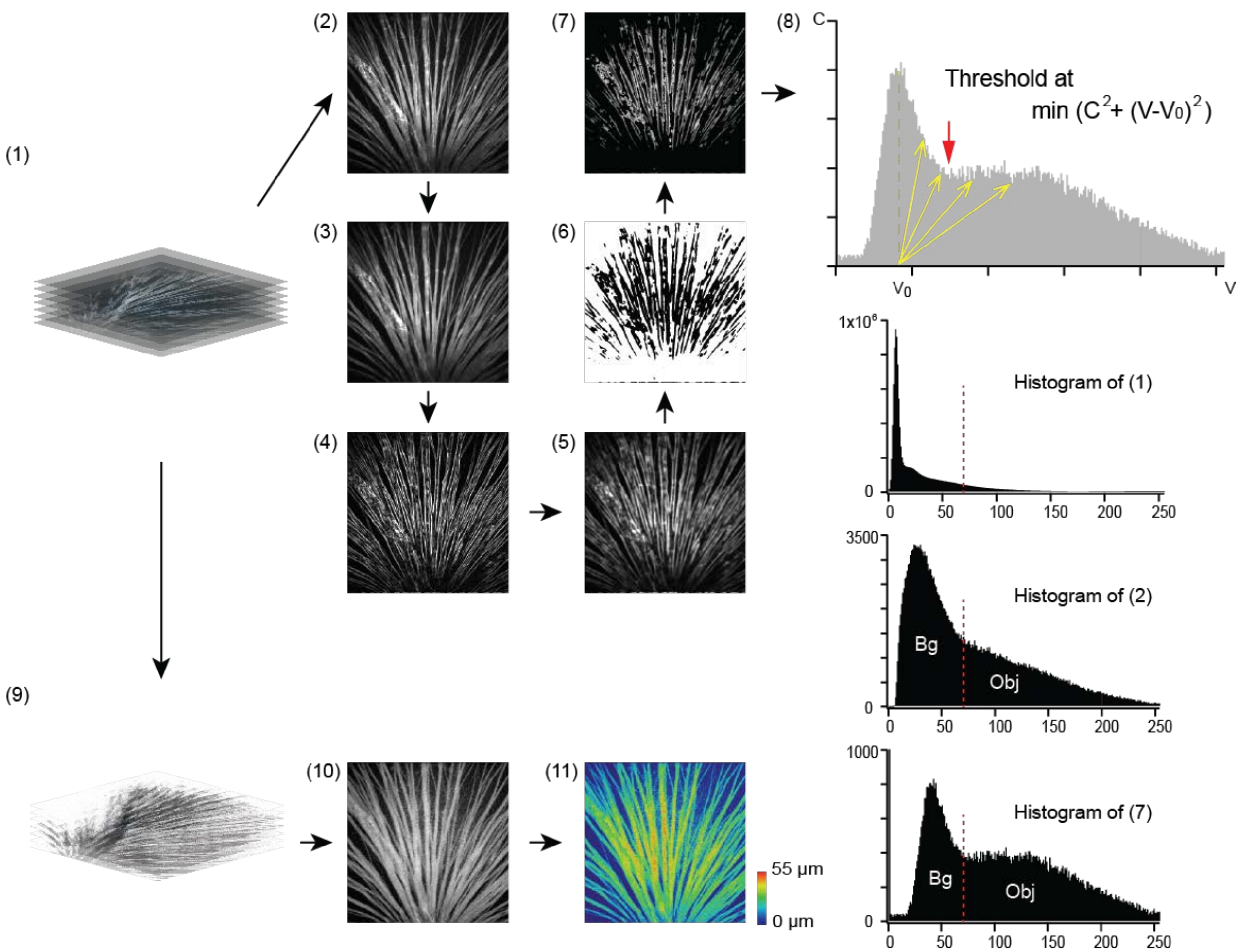

Figure S4. Morphometry by automatic thresholding

1) Take an average projection of background-subtracted, polarization-combined z-sections.
2) Smooth by median filtering.
3) Apply Sobel filtering to detect the border of nerve fiber bundles.
4) Expand the width by Gaussian blur (‘dilation’).
5) Convert it into binary to obtain a border mask.
6) Apply the border mask to the average projection, selecting near-border pixels.

7) Produce the histogram of near-border pixels.
8) Apply Triangle method to find a threshold value distinguishing the nerve fiber bundles (Obj) from background (Bg).
9) Threshold the z-sections into a binary z-stack.
10) Take a sum projection of the thresholded z-stack.
11) Multiply with the step size to obtain a 2D map of thickness.

Reference

1. Sharoukhov D, Lim H. On probing conformation of microtubules by second-harmonic generation. *Journal of Modern Optics* 2016;63:71-75.