Differentiation of biomolecules/fluorophores using spectral TPM.

Figure S1-1A shows the images acquired by each of the 32 PMT channels in black and white. Each of the 32 PMT channels has a spectral bandwidth of 8.9 nm and the total spectral range covers from 415 to 690 nm. To analyze the raw data, each channel is assigned a unique pseudo-color (ranging from dark blue to deep red). A true-color image that represents all emission spectra can be created by combining all 32 'pseudo-color' channels. An example is shown in Figure S1-1B.

Using the true-color rendered image, fluorescent molecules can be differentiated based on the rendered color even though these fluorescent molecules may be spectrally overlapped to some degree. For example, the images shown in Figure S1-1 were acquired from the sclera near the TM region. The collagen of sclera emits a narrow SHG signal in the 459 and 468 nm channels (Figure S1-1A) and is represented as 'blue' in the true-color image (Figure S1-1B). The melanin pigment in the ciliary body has a very broad fluorescent spectrum (from 490 to 690 nm) and combination of these multiple pseudo-colored channels renders the ciliary body 'redish' in the true-color image. In this eye, the anterior chamber was perfused with yellow polystyrene beads that fluorescence between 539 to 619 nm; however these channels overlap with the melanin fluorescent spectrum. In traditional multiphoton fluorescent microscope using a standard fluorescent filter, the fluorescent signal of the beads will be erroneously captured as the fluorescent signal of melanin. However, using the 32 channel PMT detector, the fluorescent beads (yellow) can be differentiated from the melanin (redish) in a true-color image (Figure S1-2B.

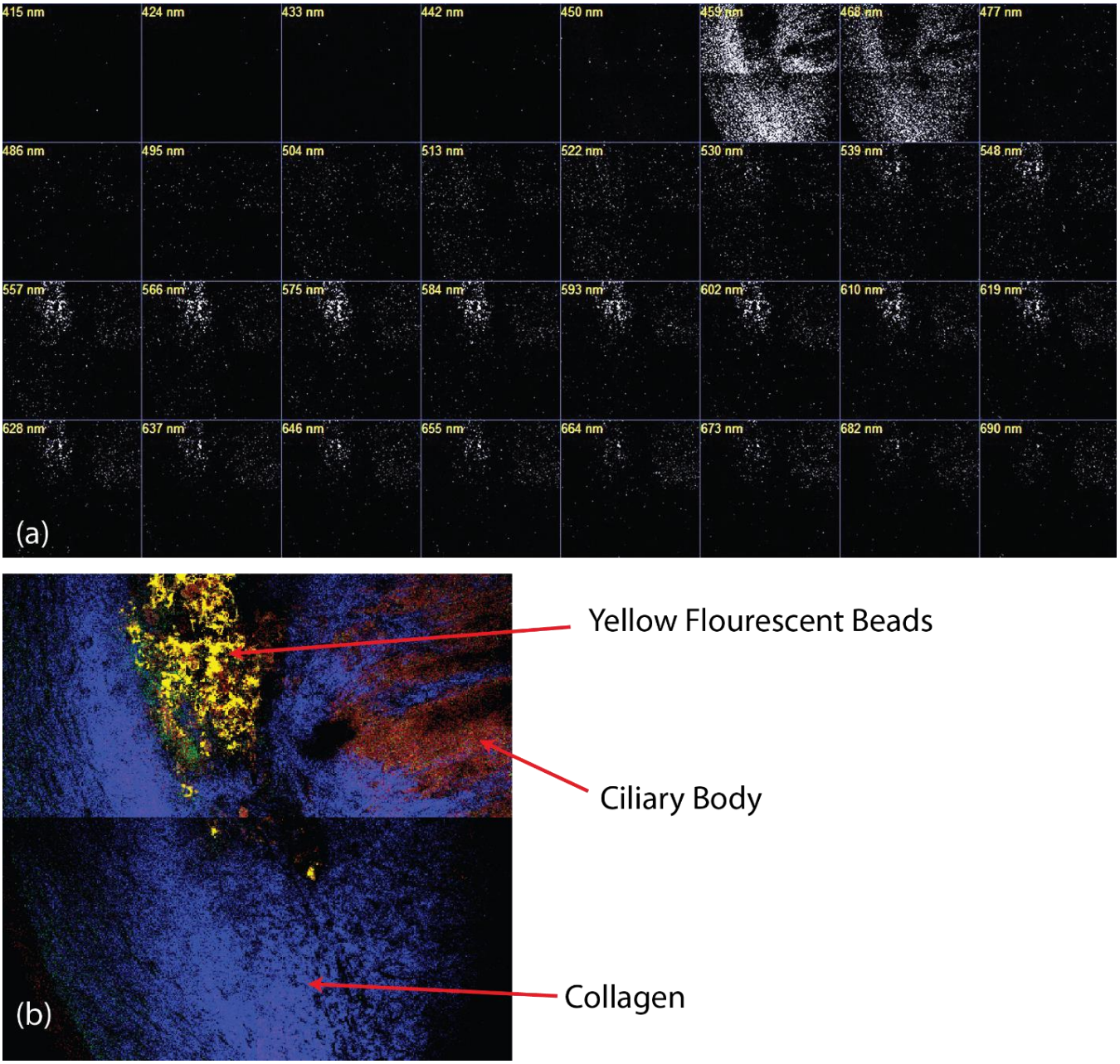

Figure S1-1: (A) Black and white image of the 32 PMT channels obtained from the spectral detector. (B) The true-colored imaged rendered as a combination of the 32 'pseudo-colored' PMT channels. Different fluorescent biomolecules/fluorophores can be identified based on their resulting colors.

Image segmentation for 3D model rendering

Several steps were involved to perform image segmentation for 3D model rendering (illustrated in Figure S1-2). 1) First the measured 3D image stack were converted into 8-bit gray-scaled images; 2) A Gaussian spatial filter was applied to the gray-scaled images to remove spatial high-frequency noise for better contouring accuracy; 3) Using an image threshold (pixels in the lowest 10% intensity value), contours were drawn around voids to delineate the AOS: scleral collagen boundaries; 4) Black-and-white images were created using the drawn contour; 5) 3D structure based on the high contrast images were used to reconstruct and render the three-dimensional model using the 3D visualization routines.

In analyzing the raw image stacks we determined that the scleral tissue contains different sizes of voids. The selection among these voids for further segmentation analysis was based on 1) size and 2) continuity. In general, only voids with a single dimension > 10μm were selected for contour analysis. In addition, only voids that formed extended continuous connections were selected for further segmentation analysis. The SC was largest of these fluid-filled spaces, identified by its relatively large size and its location near the anterior chamber at the insertion point of the IR and the TM. Voids were categorized as CC in this study based on their relative larger size, and having a tube-like structure that more or less branched straight from SC toward the surface of the eye. AVs were similarly sized fluid-filled structures located closer to the scleral surface. Other smaller voids, however, may form an interwoven network in the scleral tissue but these voids typically did not appear to connect between AV and SC. These smaller voids were excluded from 3D model reconstruction and size measurements in this study.

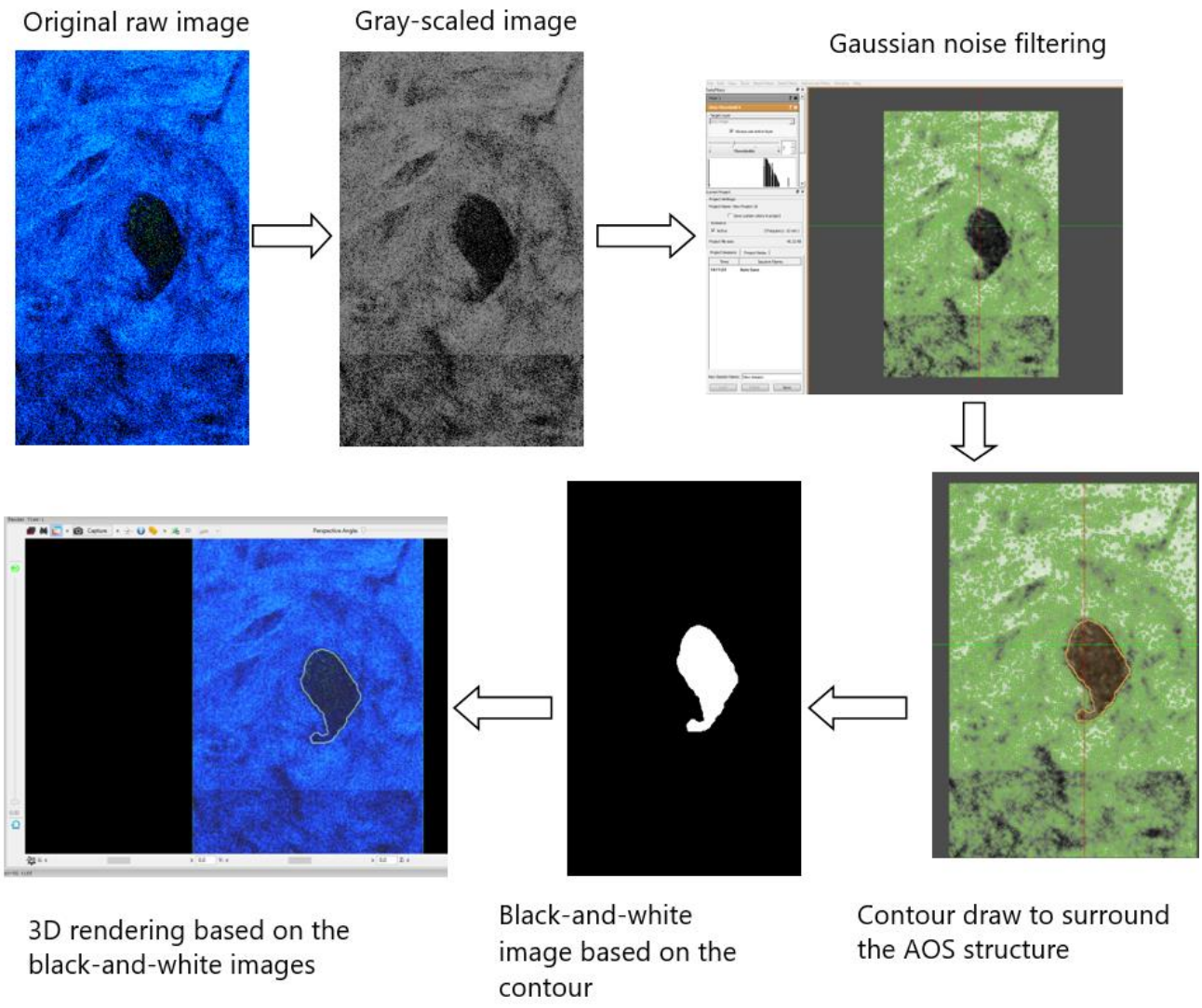

Figure S1-2: The procedure of processing the images acquired using spectral TPM to create 3D model of the AOS.