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Research report

# High resolution three-dimensional reconstruction of the collagenous matrix of the human optic nerve head

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ABSTRACT

Glaucoma is the second most common cause of blindness worldwide, leading to irreversible loss of vision. Prior studies indicate that ocular pressure-induced displacement of the lamina cribrosa (LC) may be responsible for retinal ganglion cell axon damage inside the neural canal. We present a novel approach to imaging the entire lamina cribrosa and the scleral canal at high lateral and axial resolution by using a combination of array tomography and nonlinear optical imaging of serial ultrathin orthogonal sections to detect second harmonic generated (SHG) signals from collagen. The resulting images can be analyzed individually or combined to form a three-dimensional reconstruction of the lamina. Due to the specificity of SHG generated from collagen the density and distribution of collagen inside the scleral canal can be objectively quantified with a high degree of accuracy. The reconstruction shows a non-uniform distribution of collagen along both the longitudinal and orthogonal axes. Mapping the collagen density by geographic region reveals significant differences in collagen content that result in “thin spots” with low collagen density as well as areas of very high collagen content. This suggests a non-uniform mechanical stiffness across the lamina that may account for increased axon damage observed in glaucoma patients. The inferior temporal region of the ONH in particular is marked by low collagen density, which corresponds with clinical observations identifying this region as being more susceptible to damage during the onset of glaucoma. Further application of this technique will help characterize the relationship of age, race and gender on the morphology of the LC.



## 1. Introduction

Glaucoma accounts for over 12% of global blindness [28], representing a serious health problem especially considering the irreversibility of glaucoma-induced optic nerve damage. Approximately 50% of glaucoma cases are of the primary open angle (POAG) variant. Clinically, POAG is described as a progressive visual field loss [8,9] and posterior displacement of the lamina cribrosa (LC), cupping of the optic nerve head (ONH), thinning of prelaminar tissue with axonal dropout in the ONH, and death of retinal ganglion cells (RGC) [27,22].

Past studies have observed that increased intraocular pressure (IOP), a major risk factor for glaucomatous change, results in blocking of retrograde and anterograde axonal transport [19] as well as dilation of axons passing through the ONH precisely at the level of the lamina cribrosa (LC). The LC is a sieve-like structure inside the scleral canal comprised of overlapping and branching collagenous beams [16]. These collagen beams form pores through which retinal ganglion cell axons transit to exit the eye. Generally, it is thought that the lamina cribrosa provides structural support to the exiting axons and the vascular supply of the ONH, while helping maintain the pressure differential between the inner eye and the central nervous system. This has led to the theory that an increase in intraocular pressure (IOP) causes pores to deform as the lamina is displaced posteriorly [31], exerting additional stress on the axons and blood vessels which can initiate nerve cell death and subsequent loss of vision. As the primary load-bearing tissue in the scleral canal, the lamina cribrosa has therefore been thought to play a major role in the development of glaucoma [25,14]. To better understand these factors and to model the underlying processes, it is necessary to study the entire structure of the lamina cribrosa at high resolution.

Numerous studies have examined the structure of the LC using both optical [2,32,20,13] and electron microscopy [12,10,29]. Burgoyne et al. recently presented a method to reconstruct the primate

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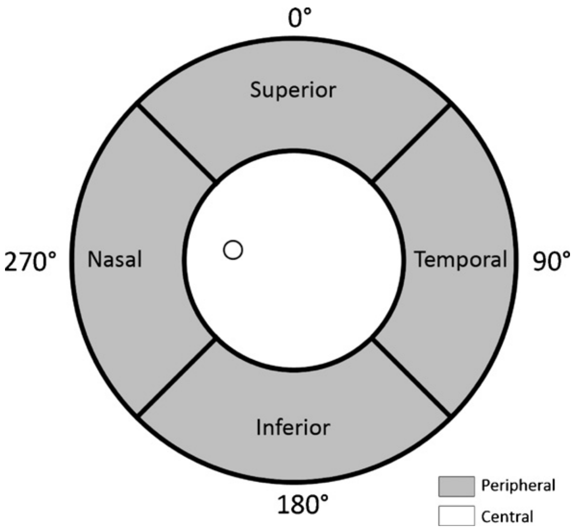


**Fig. 1.** Schematic drawing of an ONH cross-section. To analyze the distribution of collagen the ONH was divided into four peripheral sectors corresponding to anatomical quadrants. A detailed analysis for collagen content was performed by analyzing the average intensity along lines in 1° increments, starting with 0° at the 12 o'clock position.

LC using digital photography [6]. However, when examining the lamina cribrosa with traditional microscopic methods, researchers are faced with well-known problems caused by the inverse relationship between magnification and field of view (FoV). The high magnification required to resolve details of the substructure is associated with a very limited FoV, reducing the scope of the investigation and forcing researchers to focus their attention on one or several comparatively small regions of interest (ROI) that are geographically separated. While the collagen beams that make up the lamina have a diameter of a few microns, they form a larger meshwork with beam lengths and pore diameters several orders of magnitude larger than individual collagen fibers, extending far outside the FoV. Conversely, when reducing the magnification to extend the FoV to encompass the entire LC, details of the substructure such as individual beams can no longer be resolved. This "resolution gap" is an inherent problem of structural microscopy. To fully understand the biomechanical properties of the lamina cribrosa and to accurately quantify and ultimately model these properties, it is necessary to reconstruct the entire structure at high resolution to have sufficient data to understand the relationship between the finer elements that make up the tissue. Bridging the resolution gap between these scales can be achieved by acquiring multiple consecutive overlapping images and digitally combining them into a two-dimensional mosaic. A three-dimensional reconstruction can be obtained by combining 2D mosaics acquired at various depths throughout the tissue.

In recent years, nonlinear optical microscopy methods, particularly second harmonic generation (SHG) imaging, have improved to the point where they provide resolutions approaching the boundaries imposed by Abbe's diffraction limit, which specifies the maximum achievable resolution for any given optical system [1,2]. These developments have made highly detailed investigations of the LC possible [5]. Additionally, the ever increasing speed of personal computers has resulted in the widespread availability of inexpensive computing resources approaching performance levels commonly associated with supercomputers only a decade ago [11], enabling researchers to not only handle very large datasets but to also reconstruct and visualize them in 3D [3,30]. The ability to obtain high-resolution images and to process large datasets in the multiple-gigabyte range are necessary prerequisites to close the gap between scales.

In this report, we combined the high lateral resolution provided by multiphoton microscopy, the high axial resolution gained through array tomography [17], semi-automated image acquisition, and advanced data processing to bridge the resolution gap and image the LC at very high resolution. We applied this method to a human donor eye from a 53-year-old Hispanic male. Analyzing the resulting images for relative collagen content revealed a

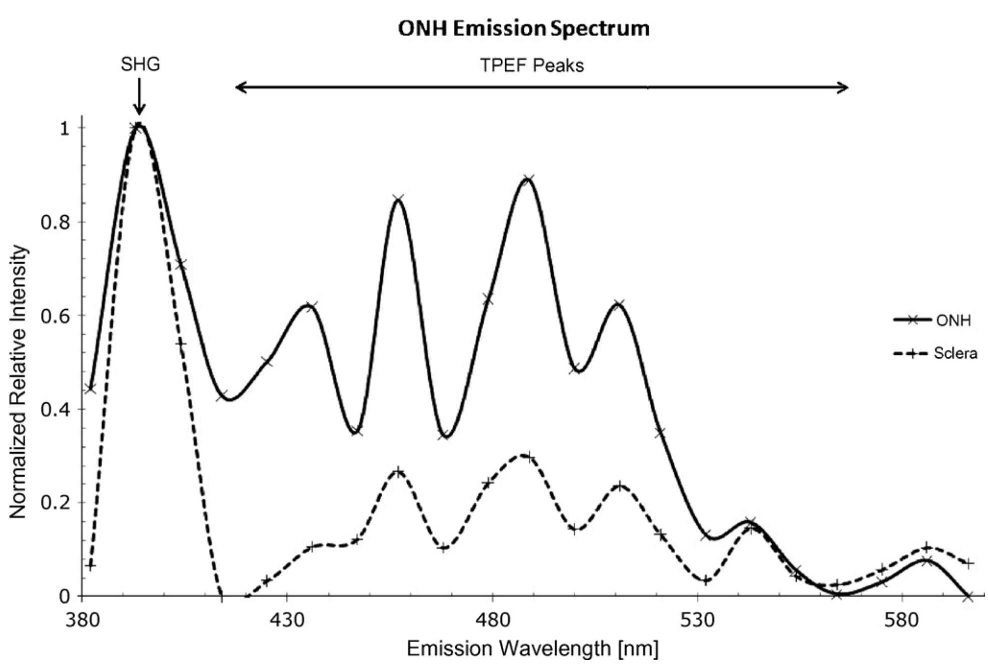


**Fig. 2.** Emission spectra of the sclera and a plastic section of human ONH tissue. The solid line shows the emissions generated from the ONH using an excitation frequency of 800 nm. The arrow marks the second harmonic peak at 400 nm. Secondary peaks at higher wavelengths indicate the presence of two photon excited fluorescence from other ONH components. Two-photon emissions from the sclera are indicated by the dashed line. Note the higher relative intensity of the SHG peak relative to the TPEF peaks. Secondary peaks match those of the ONH spectrum, suggesting a similar makeup.