

Sum-frequency generation characterization of collagen I fibers

Synopsis: Collagen is the most abundant protein in the human body, where it is a key component in connective tissue such as skin, cartilage, tendons and bones. Collagen is a polypeptide that assembles into triple helix structures that organize into fibril, which, in turn, congregate into fibers. The microscopic structure of collagen is non-centrosymmetric, which means that it can be visualized with second-order optical techniques.^[1] Collagen I fibrillar structures have been extensively studied with second-harmonic generation (SHG) microscopy, providing great insight in morphology and distribution, as well as their disease-induced changes. However, SHG microscopy is spectroscopically nonresonant, and does not provide any information about chemical structure. To supplement morphology mapping with chemical insight, we have developed sum-frequency generation (SFG) microscopy, which is sensitive to the molecular vibrations of collagen's chemical motifs. In this proposal, I will use SFG microscopy combined with Raman and infrared spectroscopy to study the hitherto unknown effect of pH on the morphology and supra-molecular chemical structure of collagen. Successful completion of this study will reveal the structural changes to dermal and tendon collagen due to variations in pH.

Background and motivation: Collagen structure is critical for the health of connective tissues.^[1] Changes to collagen structure can compromise the integrity of the tissue. One example is the weakening of collagen I in the cornea because of compromised crosslinking between the fibers, which can lead to keratoconus, a cone-shaped cornea that causes blurred vision. Other structural changes contribute to the loss of collagen density and mechanical stiffness in skin with age. Such changes can be mapped in detail with SHG microscopy, which visualizes collagen fibrils due to their intrinsic noncentrosymmetry. SHG maps reveal great insight in organizational changes of collagen-rich tissues, yet such information is based on density and not on the chemical modifications that underlie structural changes on a larger spatial scale. Without the underlying chemical information, understanding the origin of changes to collagen remains challenging.

Our group has pioneered an alternative imaging technique, namely SFG microscopy. Like SHG, SFG relies on the intrinsic noncentrosymmetry of collagen.^[2,3] However, unlike SHG, SFG is spectroscopically sensitive to chemical motifs. This implies that chemical information can be derived from the collagen images, which goes beyond mere morphology alone. The SFG technique is sensitive to molecular vibrational modes that exhibit both Raman and infrared absorption activity. It is possible to extract information about the orientation and connectivity of specific chemical groups in the collagen fiber and study how this information changes as the structure of collagen is compromised. In the proposed study, I will apply SFG microscopy to understand how a change in tissue pH affects the structure of collagen on a microscopic level. Understanding of pH induced collagen changes forms a basis for understanding more advanced forms of collagen degradation in tissues.

Research strategy: Through my undergraduate research in the group, I have experience with the vibrational spectroscopic properties of collagen. In particular, I have developed protocols for measuring collagen fibers in a FTIR spectrometer with high fidelity. This spectroscopic information is needed for interpreting the SFG signals in the microscope. A representative IR spectrum of collagen I and a SFG image of rat tail collagen is in Figure 1. A schematic of the SFG experimental set-up is in Figure 2. Using this experience, I plan to focus on the following research goals: 1) Determine how the FTIR and Raman collagen spectra change as a function of pH, 2)

record high resolution SFG images of collagen fibers under different pH conditions, and 3) use the spectroscopic information to interpret the effect of pH on collagen structure as reflected in the SFG images.

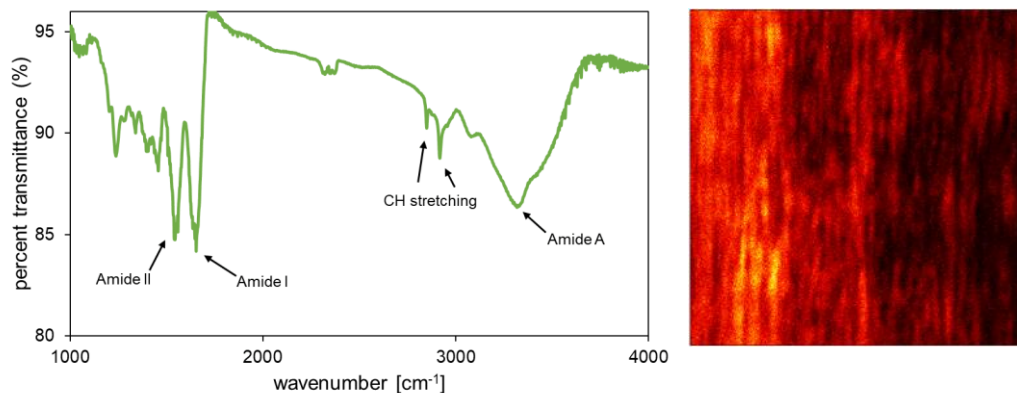


Figure 1. Left: Transmission FTIR Spectrum for Type 1 Collagen. For the measurement, a thin layer of collagen extracted from rat tail was placed between two CaF₂ windows. Right: SFG image of Rat Tail Collagen recorded at 2950 cm⁻¹ IR and 1031 nm NIR excitations.

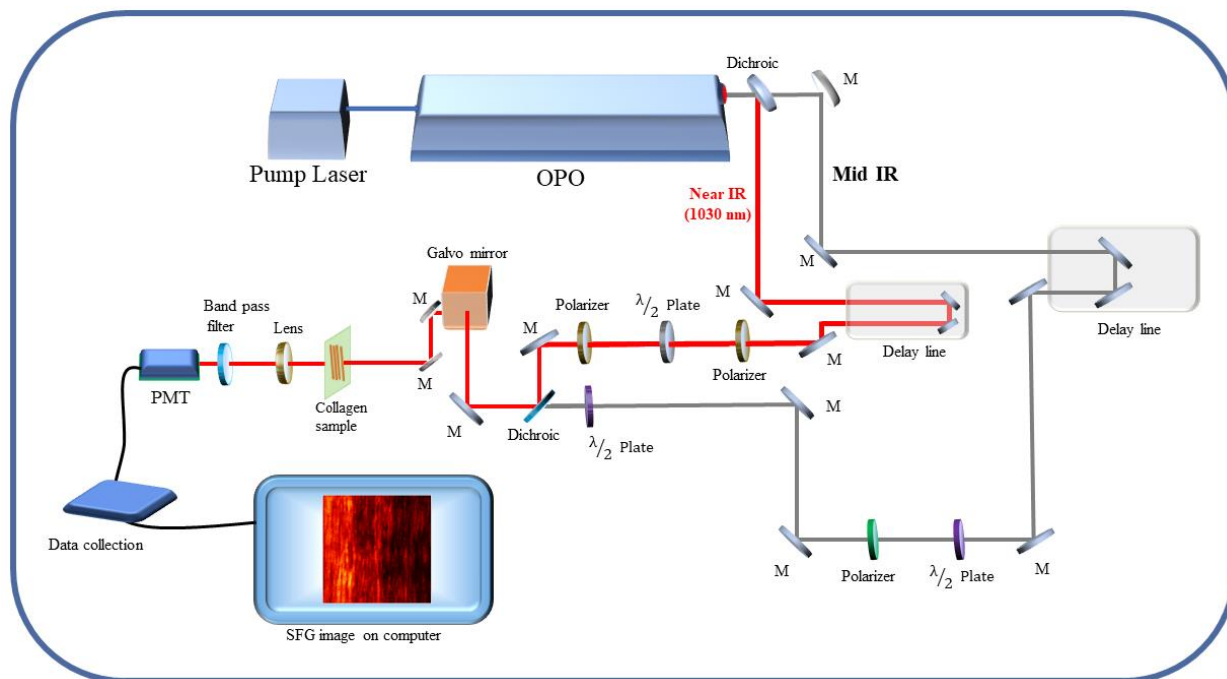


Figure 2. A schematic of experimental setup for sum-frequency generation microscopy that will be used for this project.

Timeline and responsibilities: To achieve these research goals, I will work closely with a postdoctoral fellow and under the supervision of our group's principal investigator. I will spend 25 to 30 hours per week and pursue the following steps for my SURP project:

- Weeks 1-2
 - *Prepare collagen fibers in aqueous solutions of different pH.* I will optimize conditions for preparing collagen fibers in aqueous buffers of different pH. These samples are intended for spectroscopic examination with FTIR and Raman. To reduce the effect of water absorption, I will immerse the dry collagen fibers, obtained from rat tail tendon, in buffers of D₂O, while the pH will be controlled with a phosphate buffer.
- Weeks 3-4
 - *Collect FTIR and Raman spectra of the collagen samples.* I will collect vibrational spectra of the collagen samples prepared in the previous step. For FTIR, I will first attempt to record the spectra in transmission mode by forming a thin layer of the sample between two CaF₂ substrates. Alternatively, I will use attenuated total reflection for obtaining the FTIR spectra. For Raman measurements, I will use a Raman microscope to collect spectra from small droplets of the solution deposited on glass cover slips.
- Weeks 5-6
 - *Perform SFG imaging studies on the collagen samples.* I will work with a postdoctoral scholar to record SFG images of the collagen samples. For this purpose, the collagen suspension will be deposited on CaF₂ cover slips and images will be collected within a 200 x 200 μm² field of view. We will collect images within the 2700-3200 cm⁻¹ spectral window, which corresponds to the carbon-hydrogen vibrational stretching modes. I expect that spectroscopic changes can be observed in this window as the pH level is changed.
- Week 7-8
 - *Correlating morphological and spectral changes as a function of pH.* I will perform a detailed image analysis to correlate the morphological features in the image with changes seen in the spectroscopic data. I expect that there is a strong correlation between pH-induced spectroscopic changes in the vibrational spectrum and the observed morphological features in the SFG images. Based on this analysis, I will propose a mechanistic model of pH induced structural changes to collagen I fibrils.
- Week 9-10:
 - *Literature study and scientific report.* I will perform a thorough literature study and compare my results with related results reported in the literature. I will summarize my findings in a written report. It is my aim to submit this report to a peer-reviewed scientific journal.

Conclusion. Given my experience with vibrational spectroscopy, I expect that I can complete the proposed steps listed above within the duration of the SURP program. I believe that the proposed study can contribute to a better understanding of structural changes to collagen fibrils as induced by a changing chemical environment. And ultimately, a greater understanding of collagen-related biological diseases and aging, which immensely affects society. I am very excited about this project as it allows me to use my skills as a spectroscopist and learn additional skills in optics, sample preparation, analysis and scientific communication.

References:

- [1]. Chao-Yu Chung, John Boik, and Eric O. Potma. *Annual Review of Physical Chemistry* (2013), **64**, 77.
- [2]. Yang Han, Julie Hsu, Nien-Hui Ge, and Eric O. Potma. *Journal of Physical Chemistry B* (2015), **119**, 3356.
- [3]. Adam Hanninen, Ming Wai Shu, and Eric O. Potma. *Biomedical Optic Express* (2017), **8**, 4230.