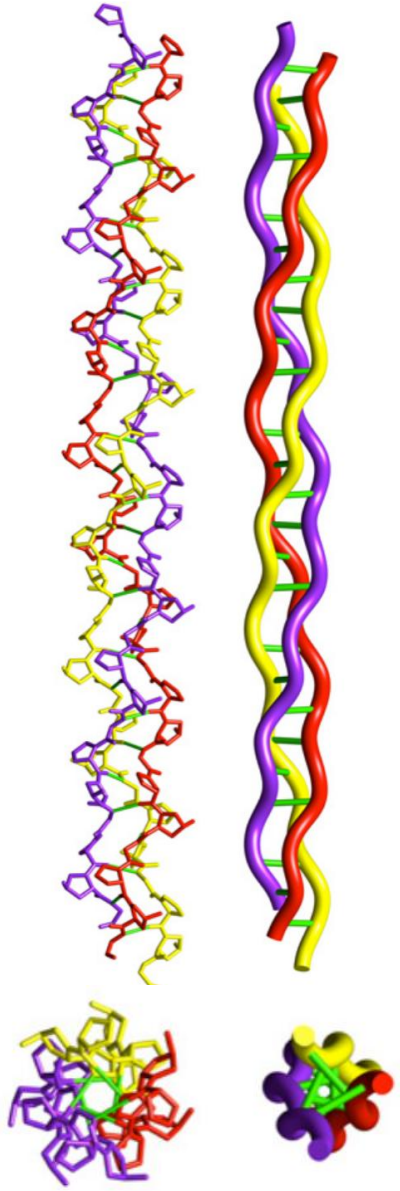
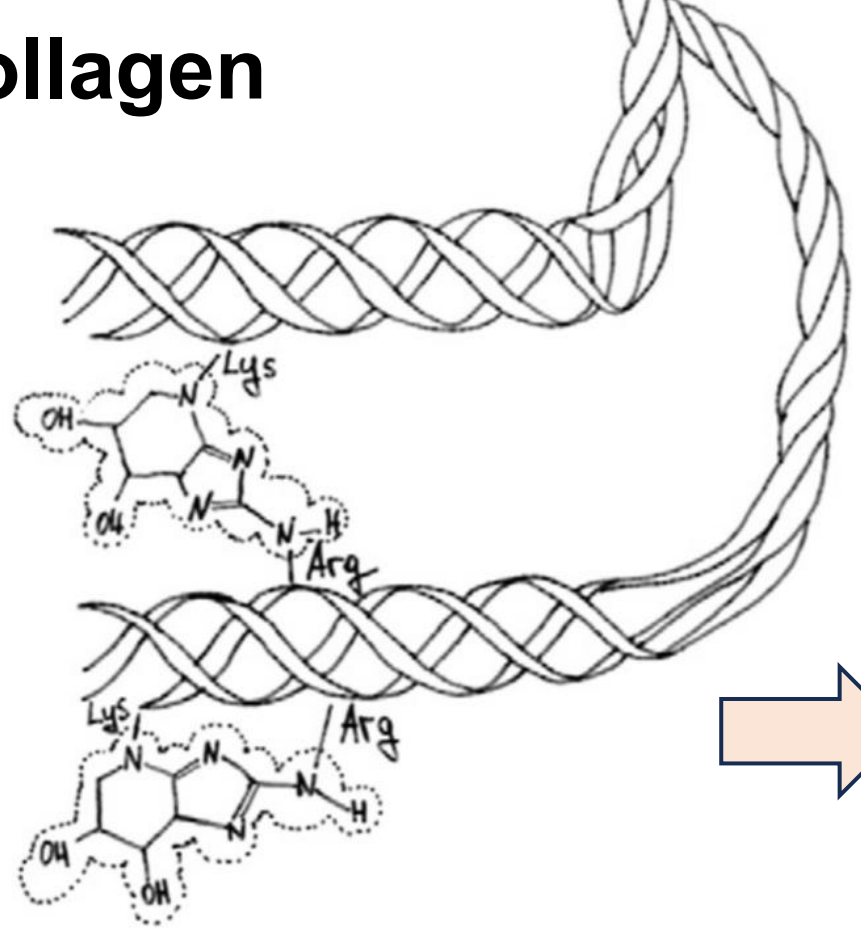
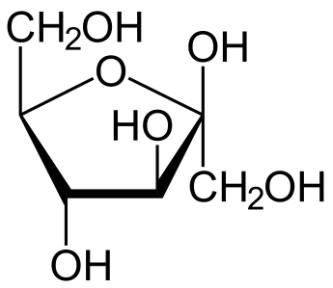
Polarization Resolved Second Harmonic Generation Microscopy Reveals Molecular Disorder in Crosslinked Collagen Fibrils

Benjamin Hansson
Richard Cisek
Danielle Tokarz
Laurent Kreplak

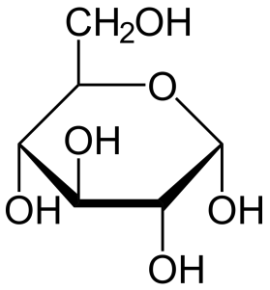
The AGEing of Collagen







Fructose

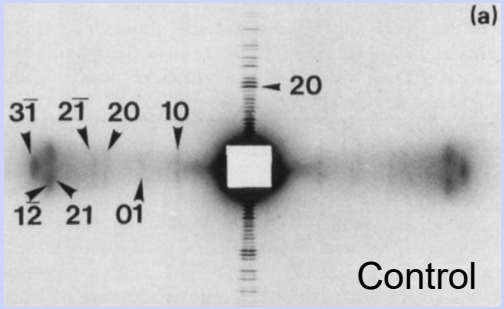


Glucose

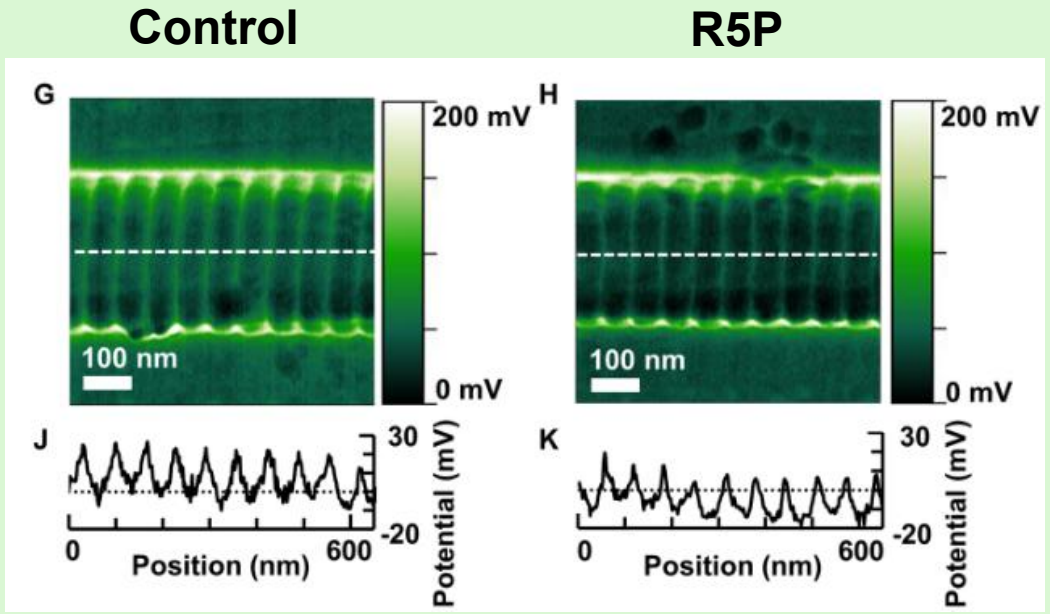
- Age-related diseases
- Cellular disfunction
- Tendon stiffening
- Impaired wound healing
- Wrinkles

Jordi Bella, Biochemical Journal, (2016)
A. Gautieri et. al., Matrix Biology, (2014)

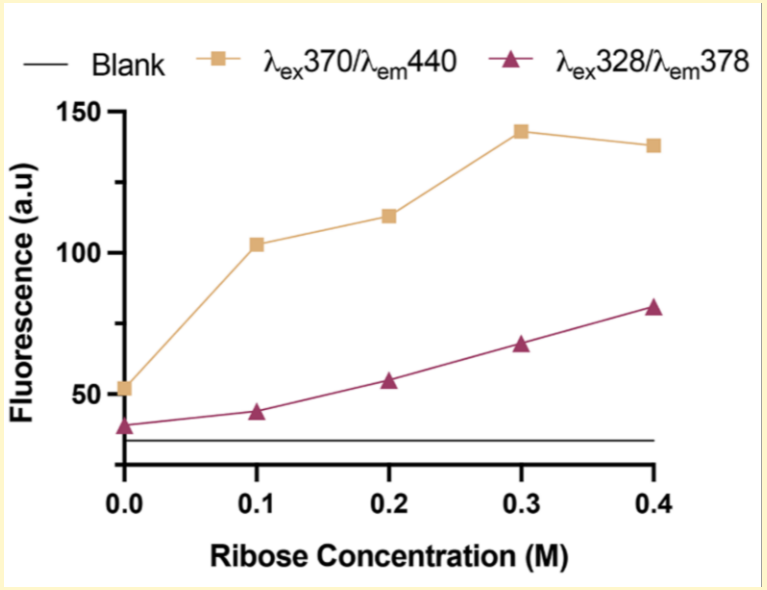
Previous Studies on Collagen Glycation



X-ray diffraction
6 weeks 0.2 M ribose
(Tanaka et al., 1988)



Atomic force microscopy
5 weeks 50 mM
ribose-5-phosphate
(Bansode et al., 2020)



Fluorescence spectroscopy
50 weeks ribose
(Sloseris et al., 2025)

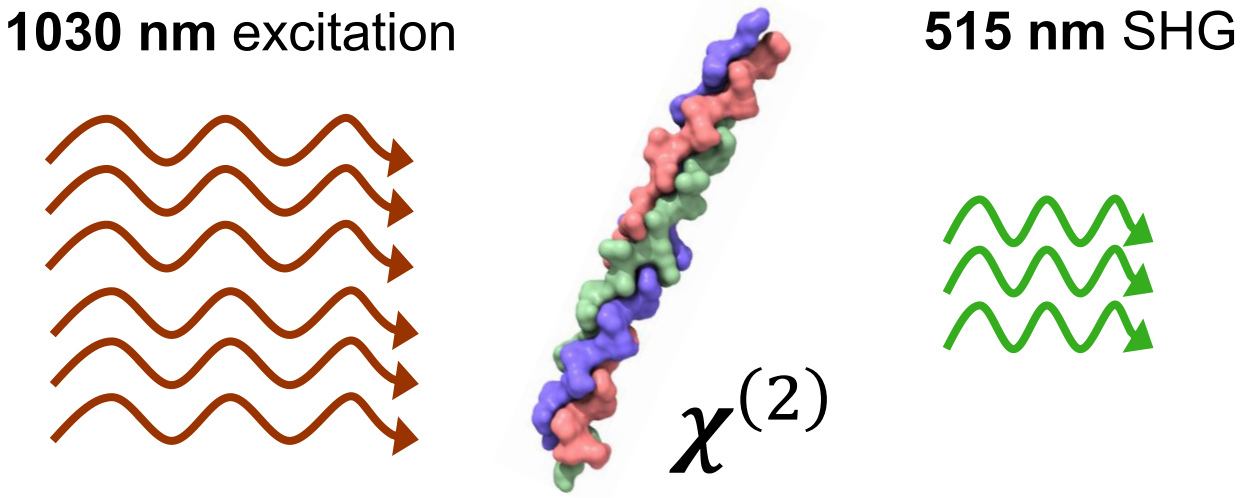
*How can we rapidly measure **nanoscale changes** in collagen structure due to glycation-induced crosslinking?*

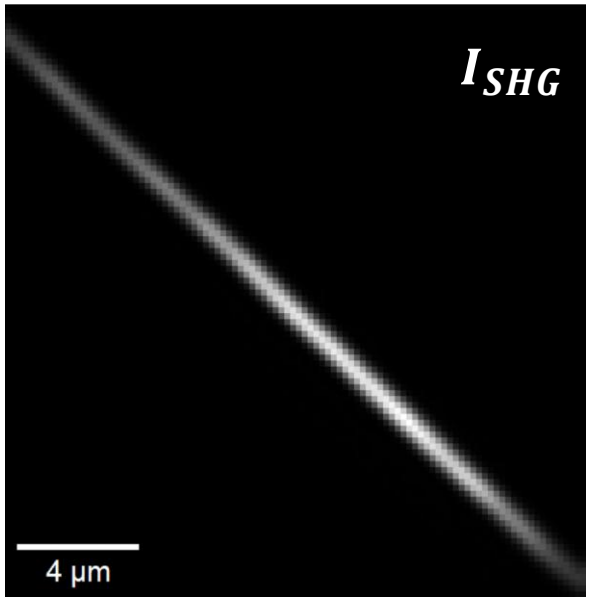
Second Harmonic Generation (SHG) of Collagen

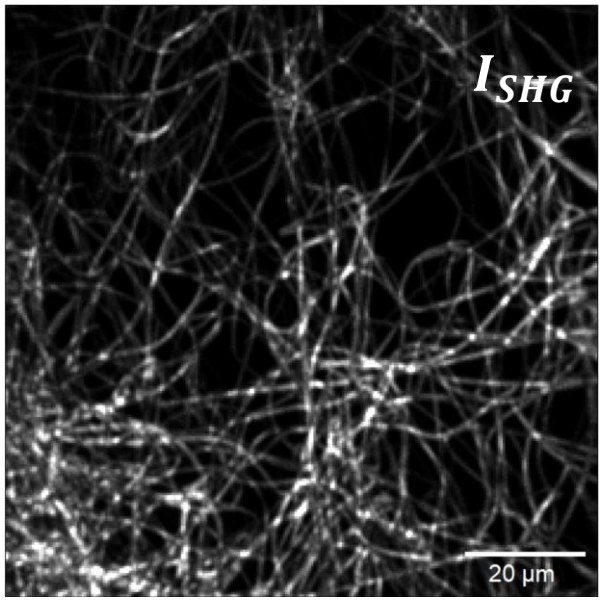
SHG occurs when two photons convert into one photon of double frequency **only** for media with non-centrosymmetric structure

$$I_{SHG} \propto \chi^{(2)} EE$$

$\chi^{(2)}$ is a material property which can be measured by analyzing the polarization of SHG light





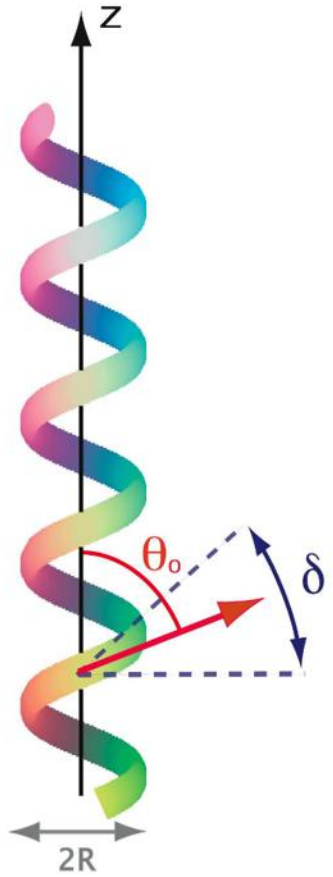


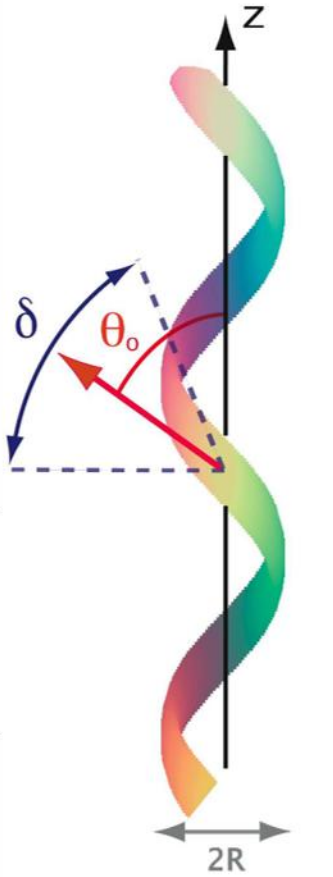
Using SHG to Investigate Molecular Structure

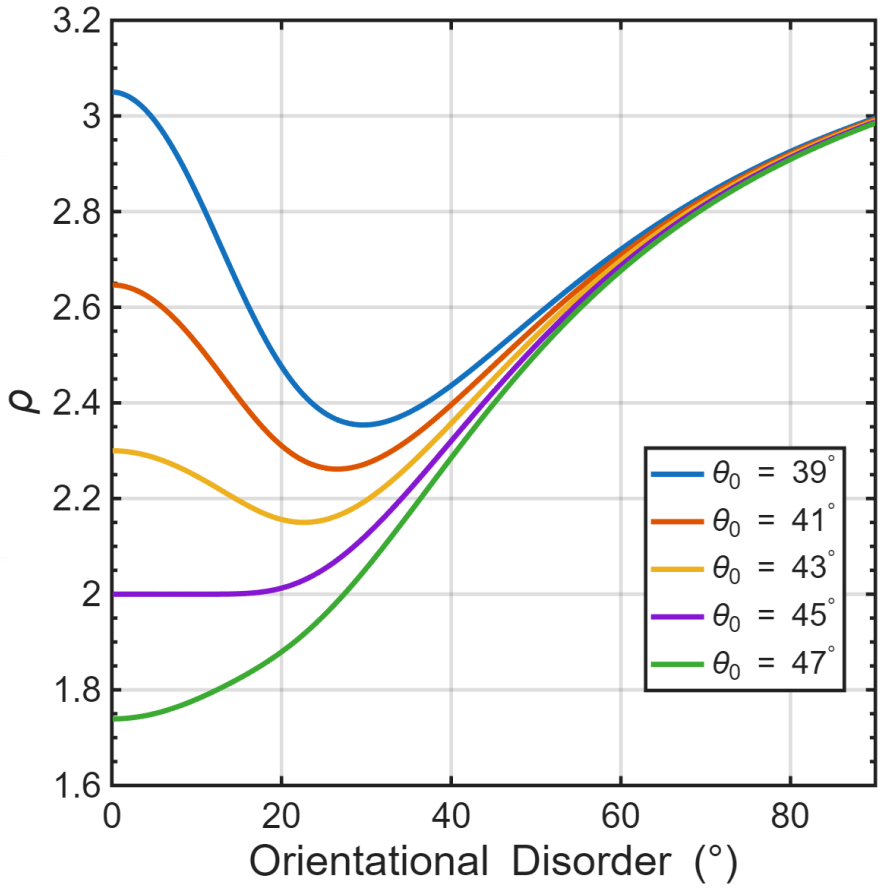
$$I_{SHG} \propto \chi^{(2)} EE$$

$$\rho = \frac{\chi^{(2)}_{ZZZ}}{\chi^{(2)}_{ZXX}}$$

$$\cos^2 \theta_0 \cong \frac{\rho}{\rho + 2}$$





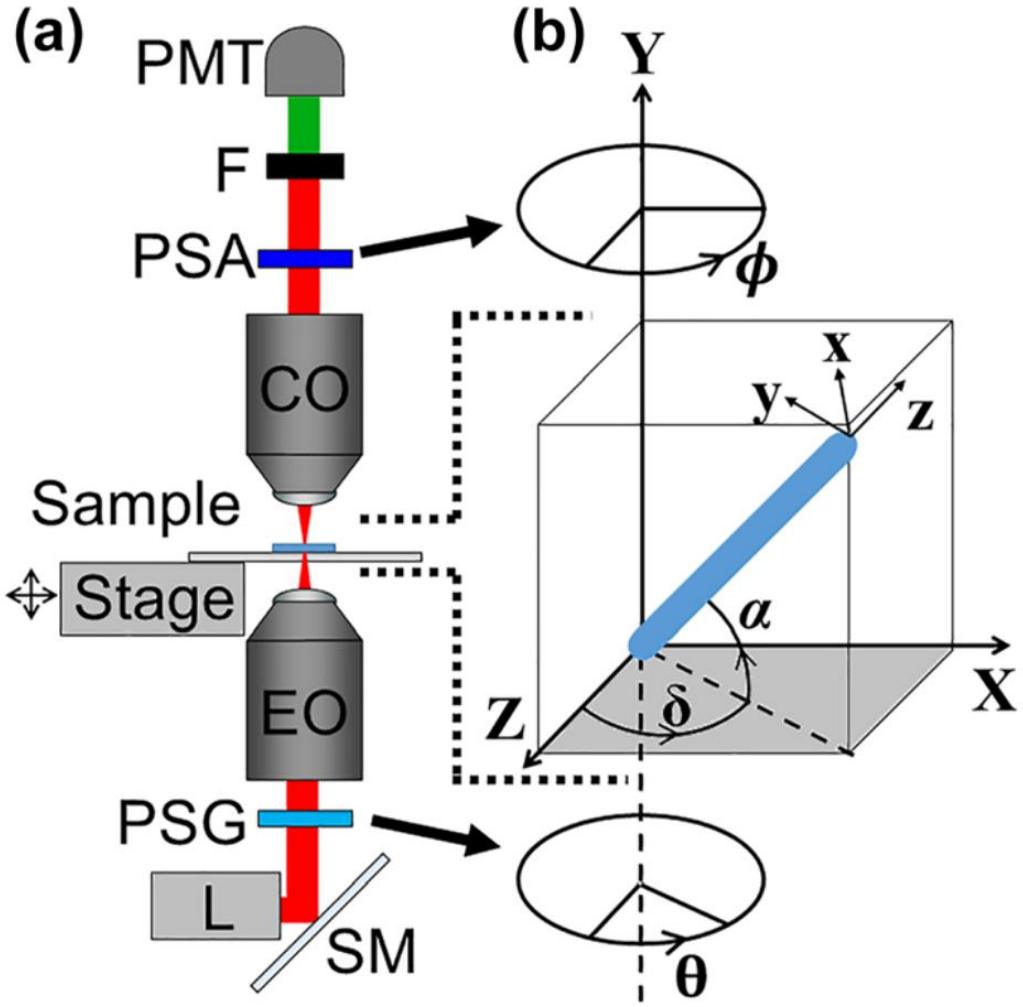


Low ρ
Compact helix

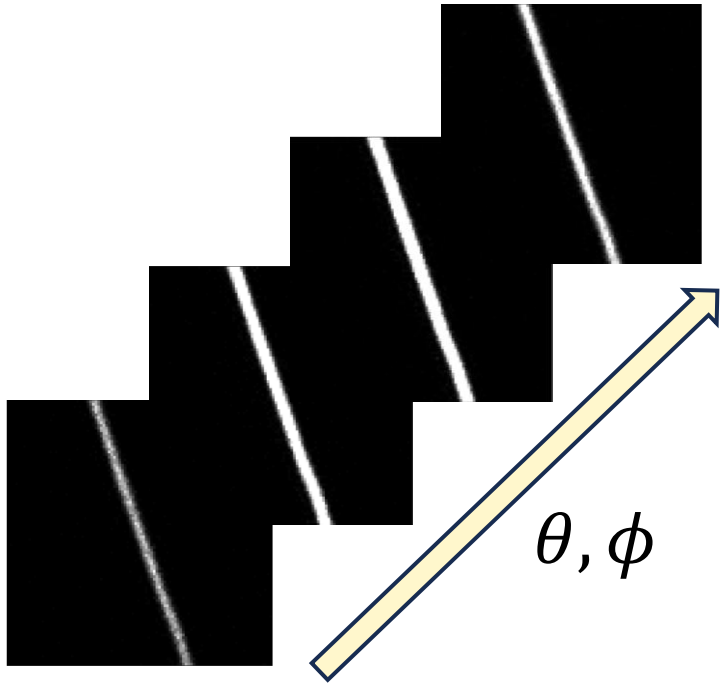
High ρ
Stretched helix

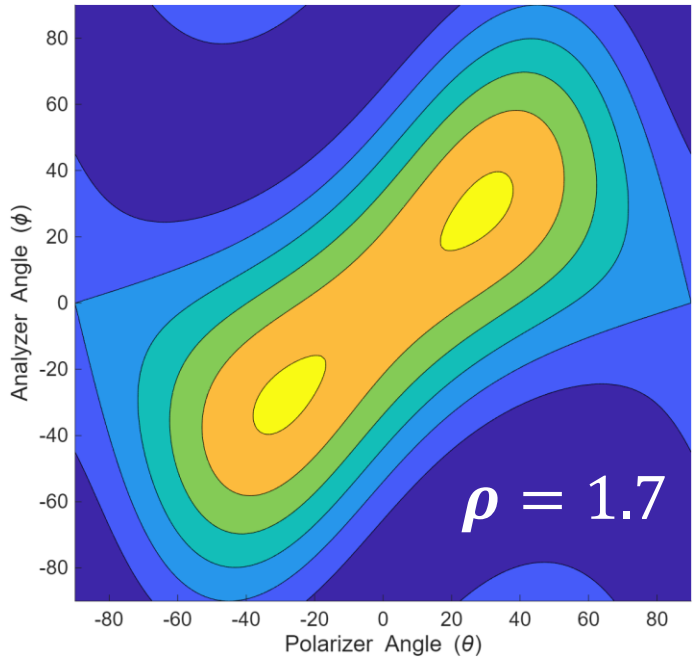
Tiaho et. al., Optics Express, (2007)

The Polarization-In Polarization-Out (PIPO) SHG Microscope



Harvey et. al., Nanophotonics, (2023)





$$I_{2\omega}^{C6V} \propto |\sin\phi \sin 2\theta - \cos\phi \sin^2\theta + \boldsymbol{\rho} \sin\phi \cos^2\theta|^2$$

Research Question 1

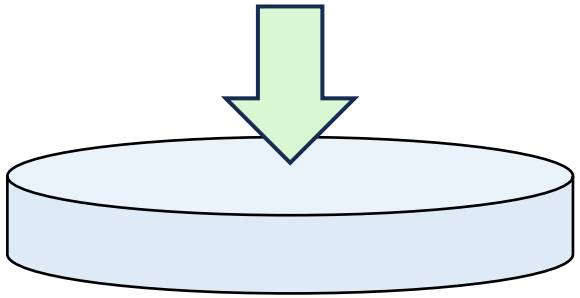
*Can **SHG** measure the difference between crosslinked and non-crosslinked collagen?*

Research Question 2

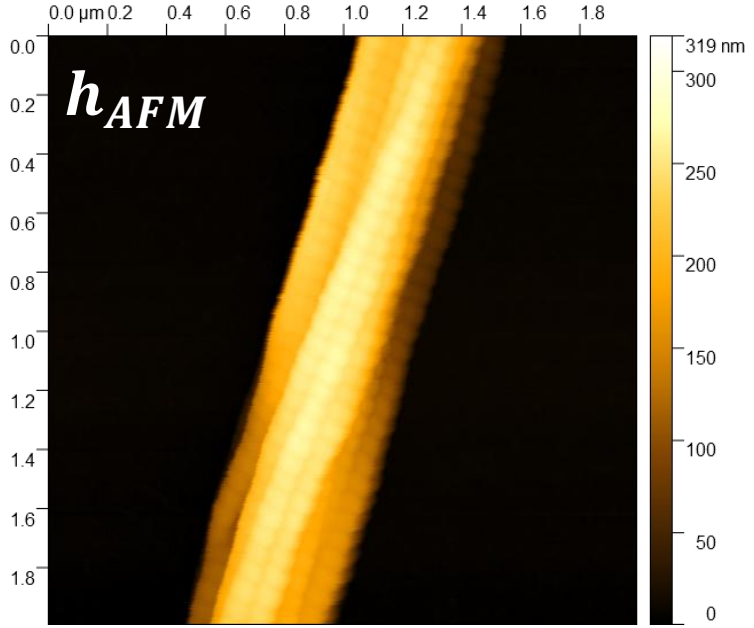
*What is the mechanism behind the glycation-induced change in collagen **molecular structure**?*

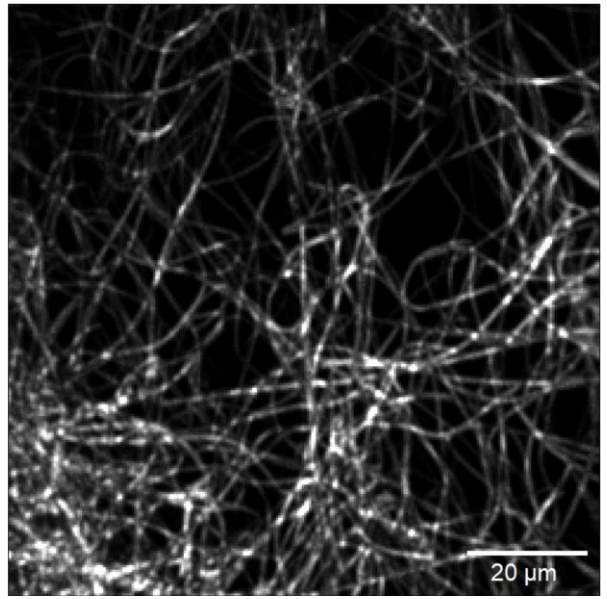
A Crosslinking-Free *In-Vitro* Fibril Platform

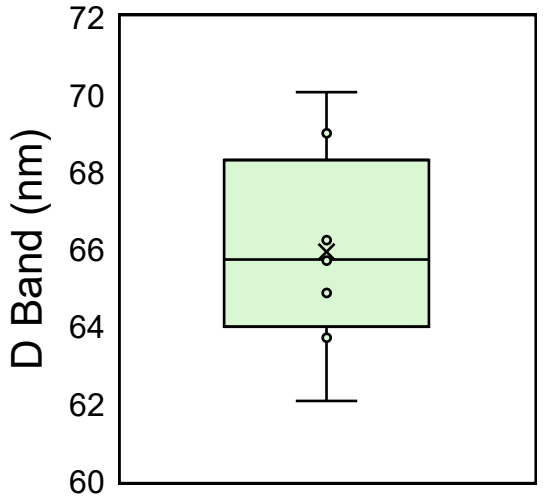
PBS
+
Bovine
Telocollagen



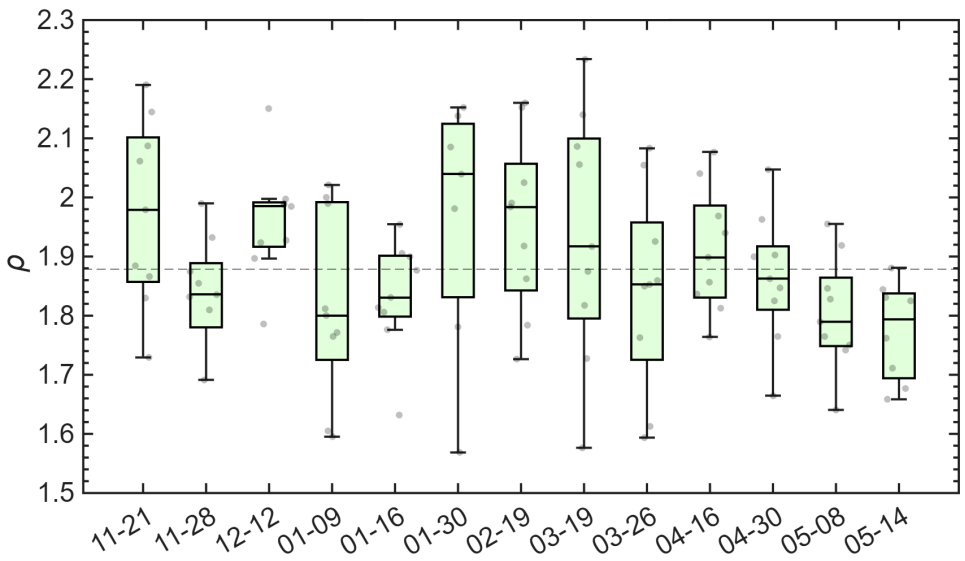
24 hours
incubation
at 30°C







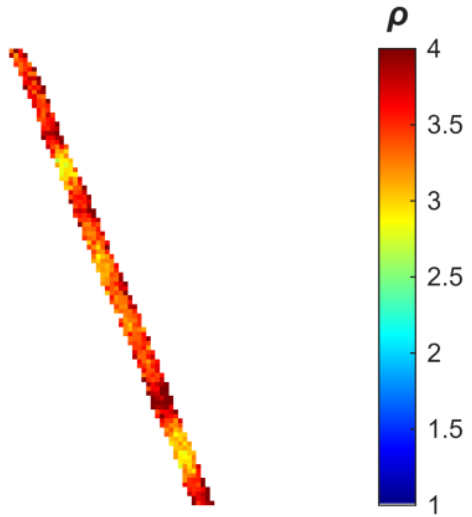
66 ± 3 nm



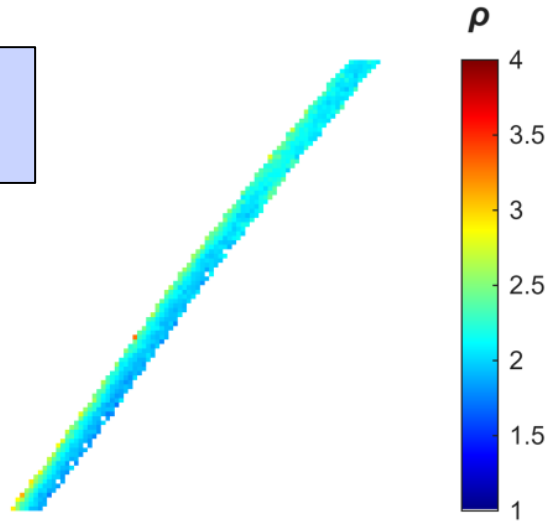
ρ = 1.9 ± 0.2

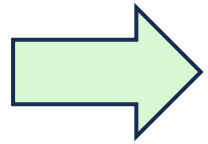
Using Glutaraldehyde to Induce Crosslinking

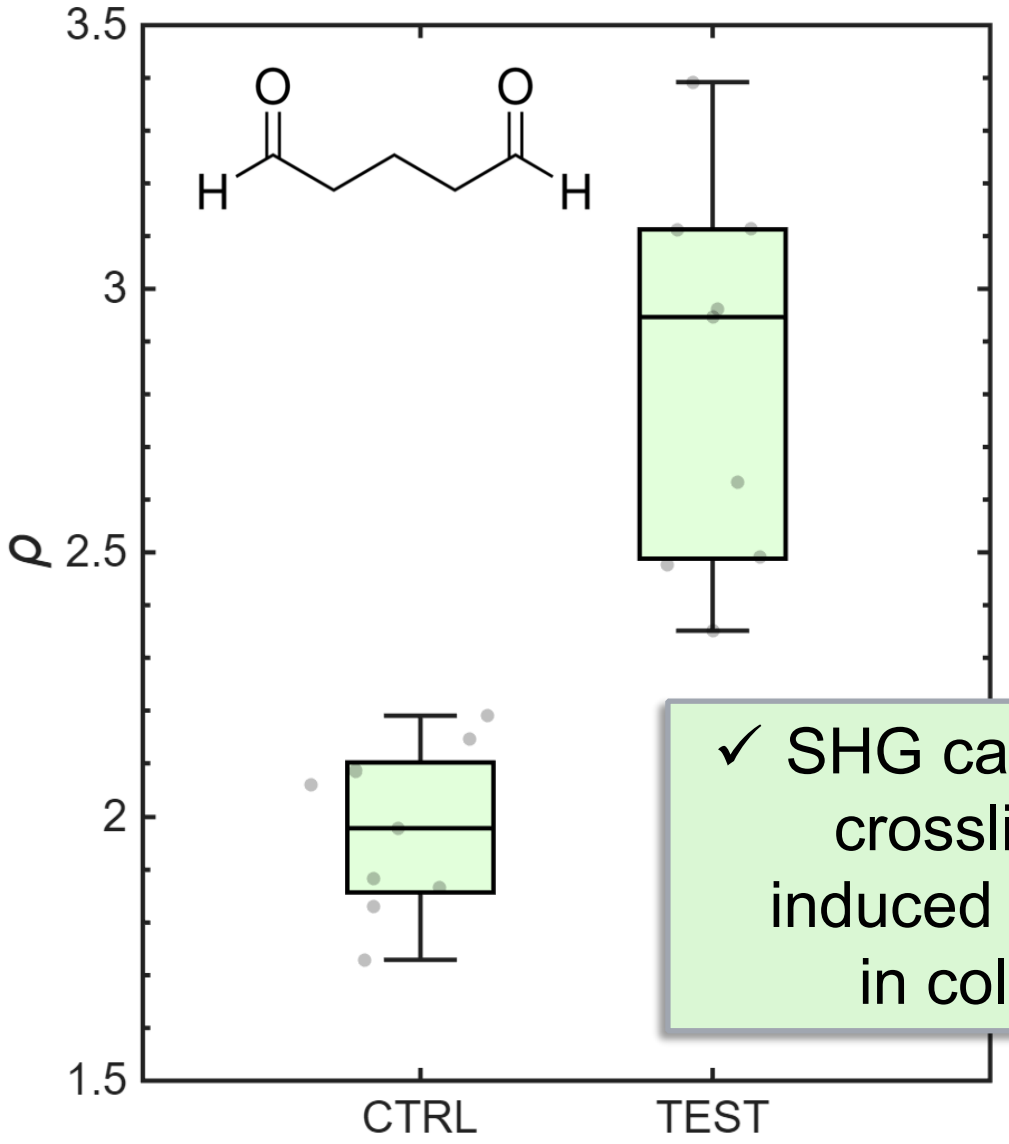
Glutaraldehyde
0.2 M 1 hour
$n = 9$



PBS Control
$n = 9$







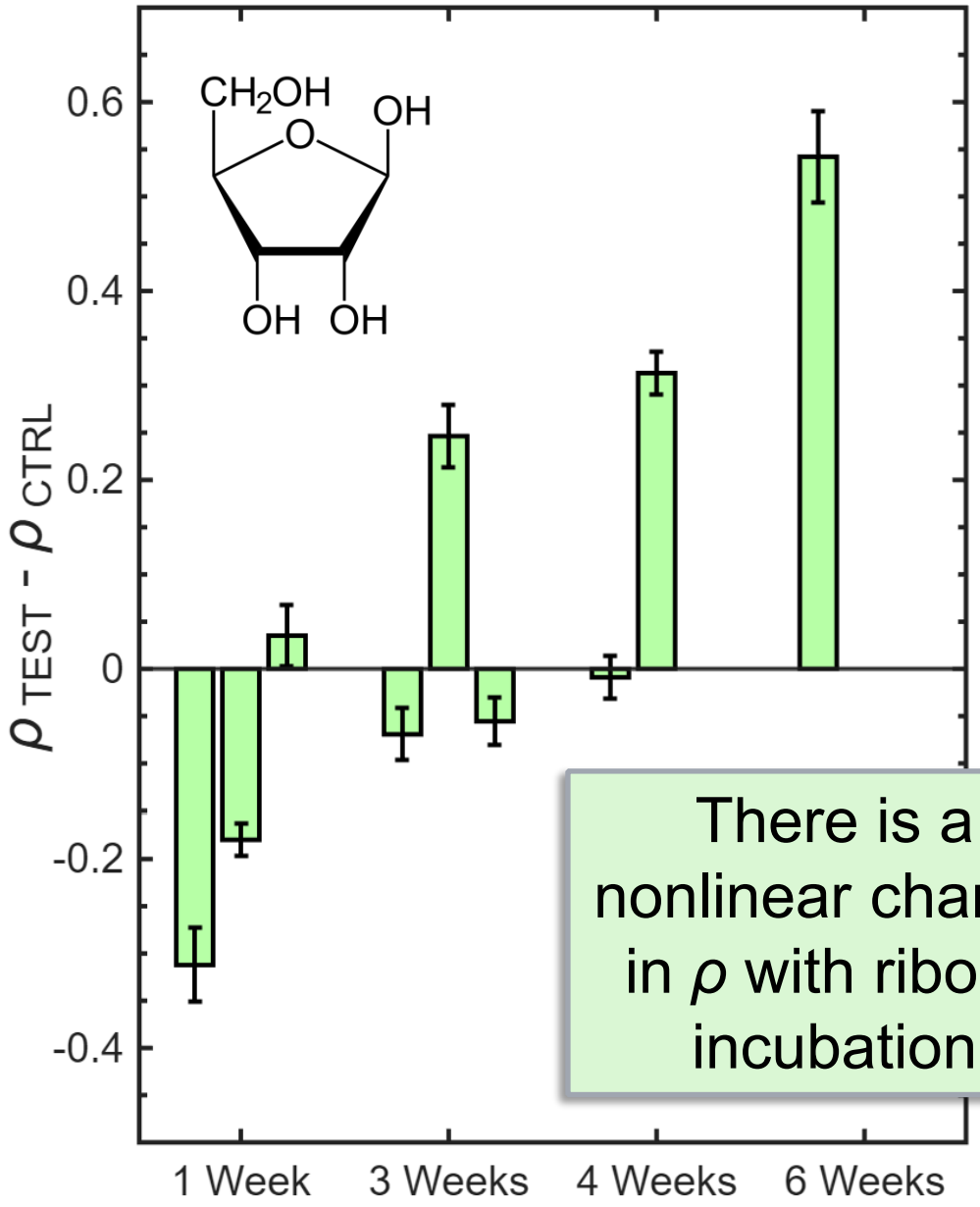
✓ SHG can detect crosslinking-induced changes in collagen

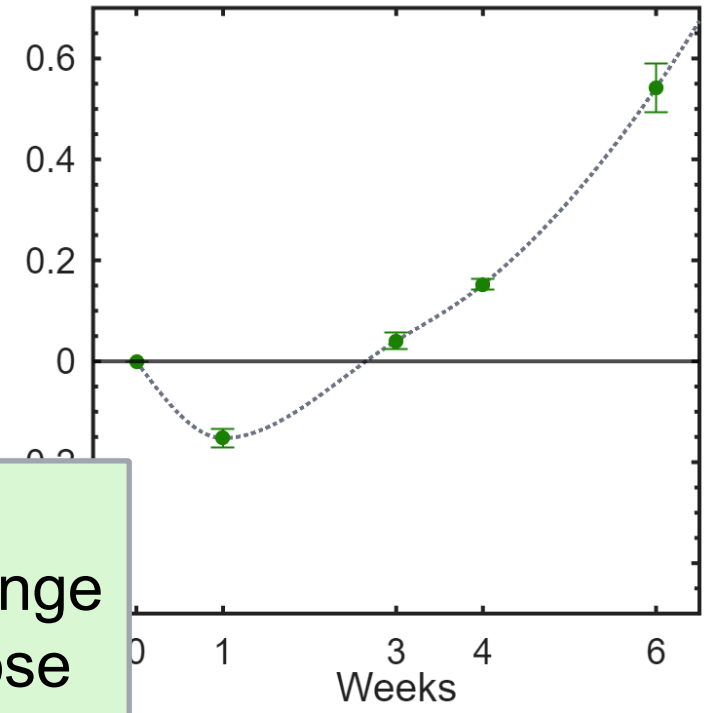
Nonlinear Change in ρ with Ribose

Goal: Measure ribose-treated fibrils at different time points to search for changes in molecular structure

Ribose 0.2 M	PBS Control

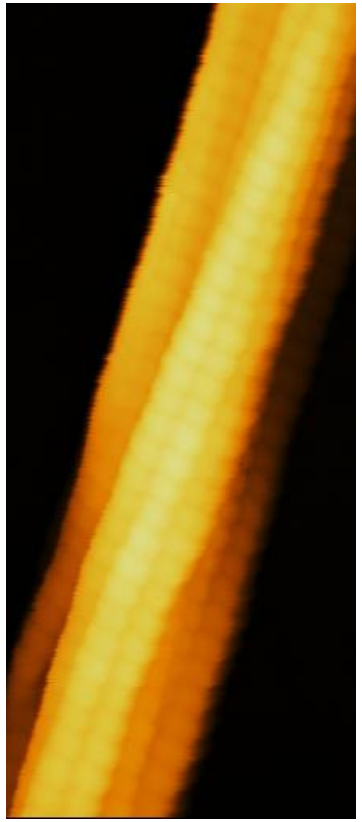
- Fresh *in-vitro* fibrils
- 1, 3, 4, and 6 weeks ribose
- $n = 9$ test and control fibrils
- Extract ρ value from each fibril, pool test and control, measure differences

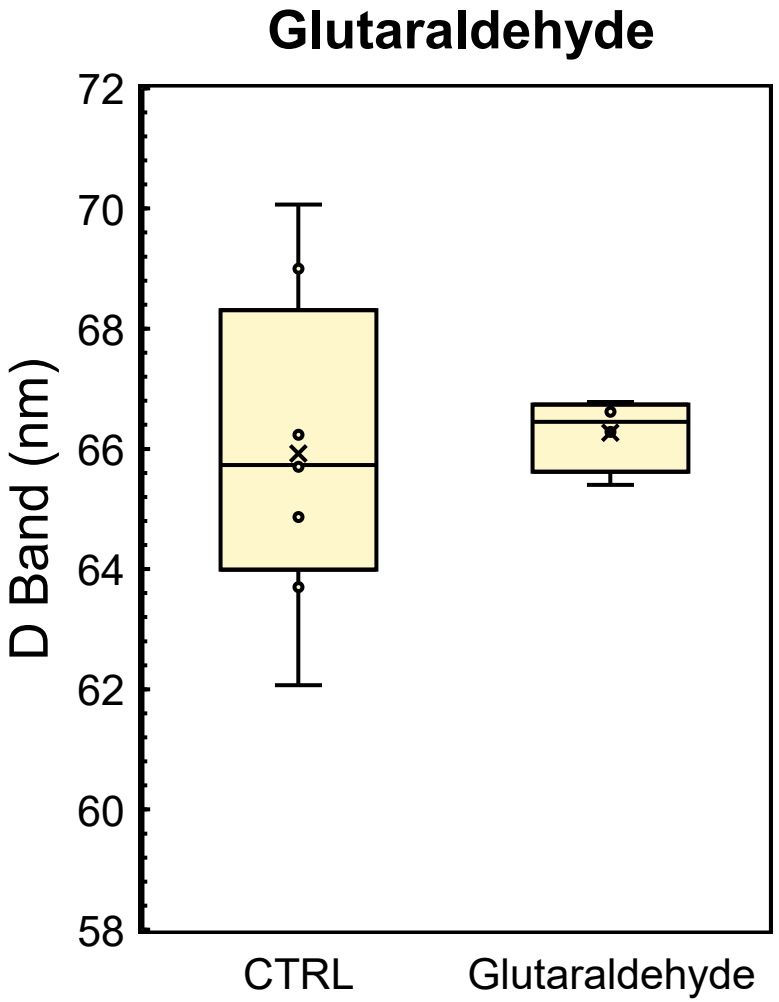


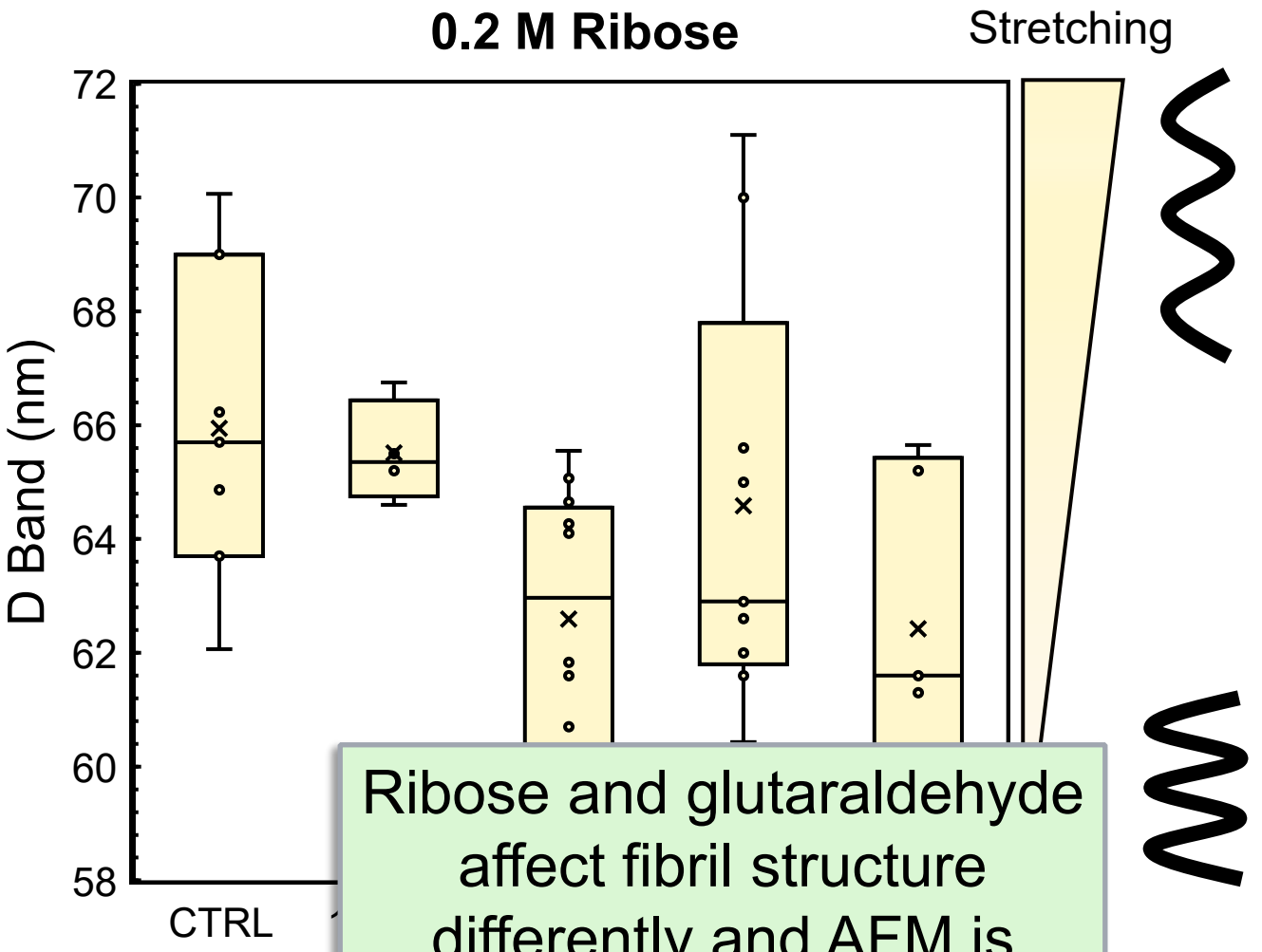


There is a nonlinear change in ρ with ribose incubation

What about the D Band?

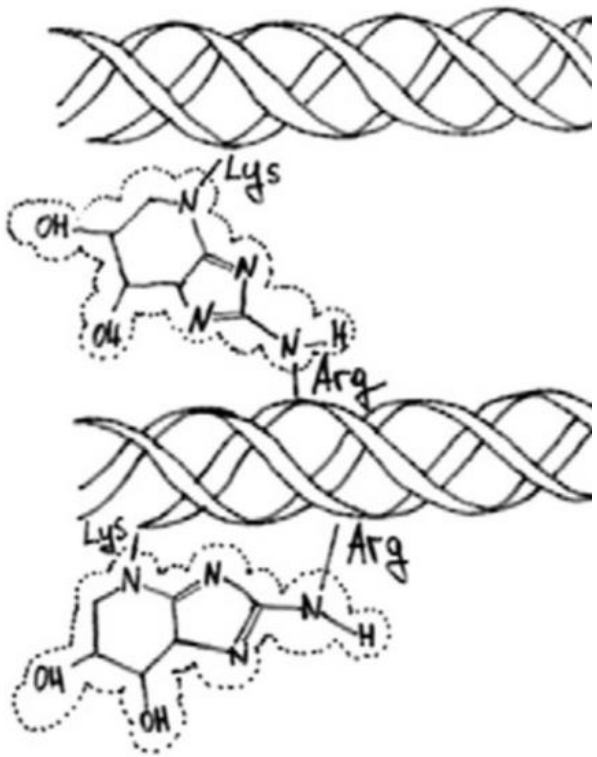




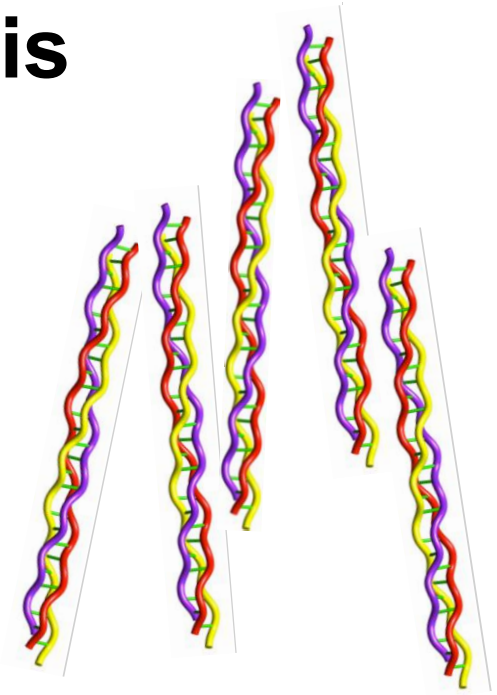


Ribose and glutaraldehyde affect fibril structure differently and AFM is insensitive to disorder

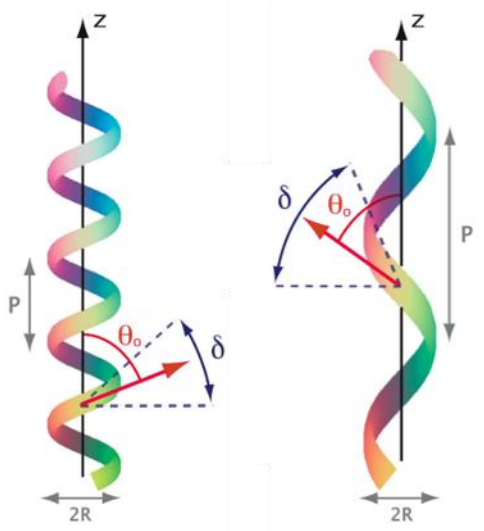
A Mechanistic Hypothesis

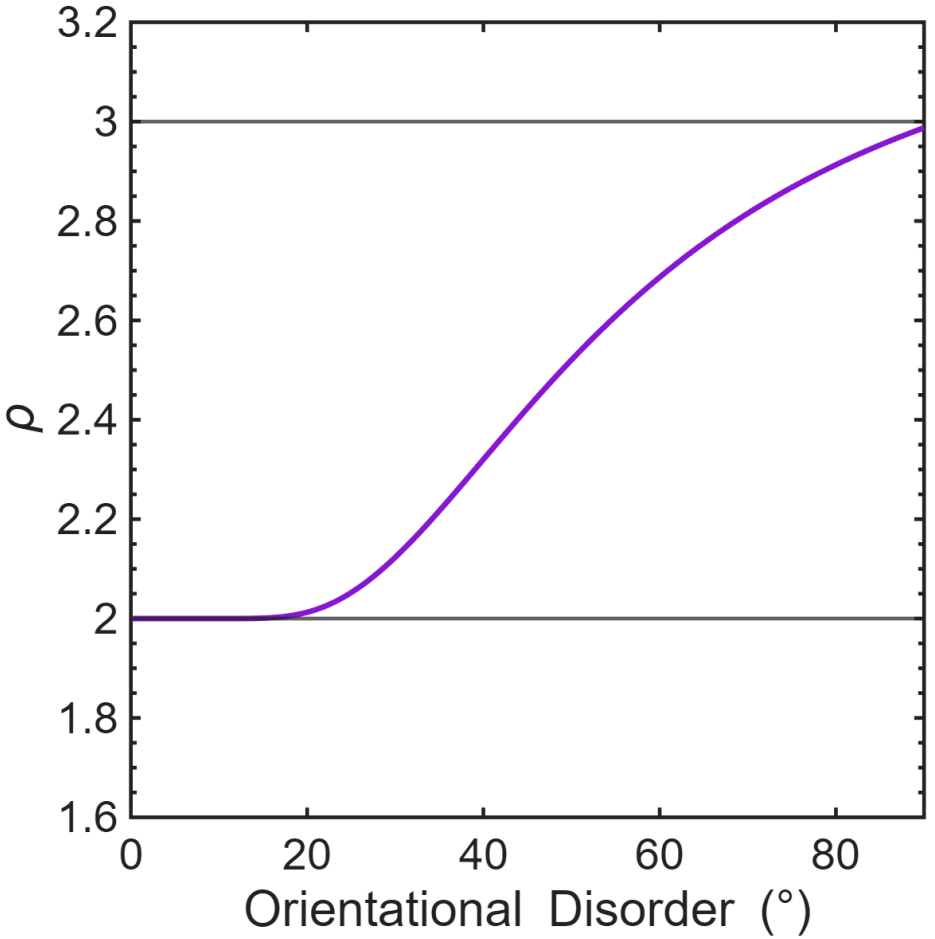


Inter
Disorder

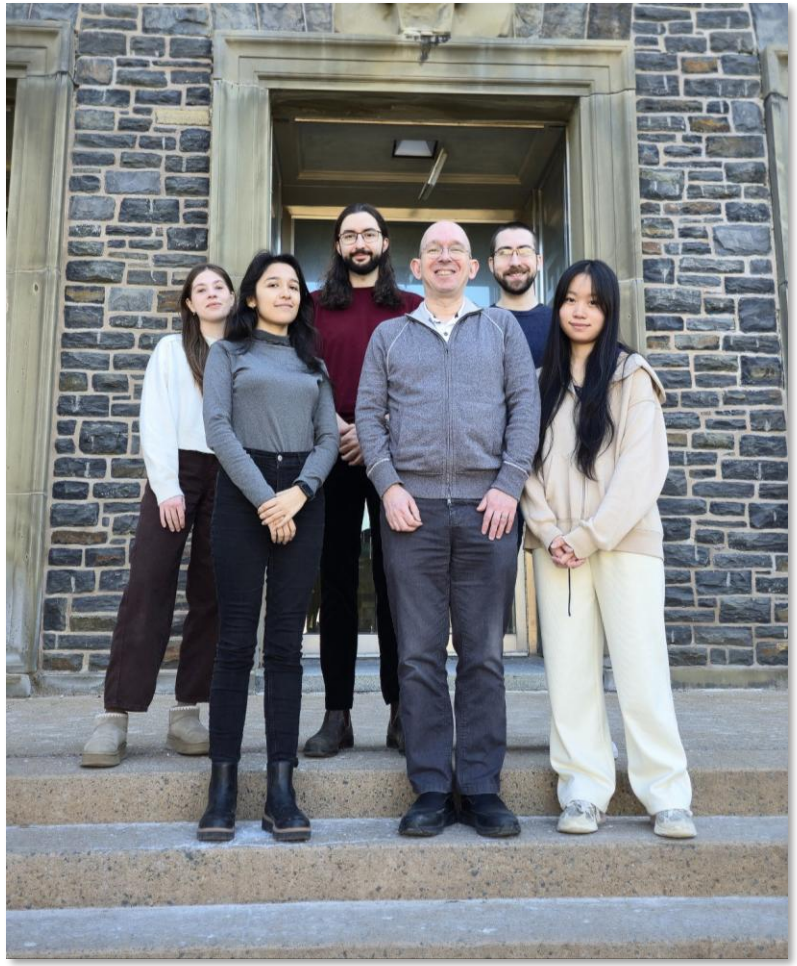


Intra
Distorts helix





Acknowledgements



- **Dr. Laurent Kreplak**
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- Sara Evans

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Ultrafast Nonlinear Spectroscopy

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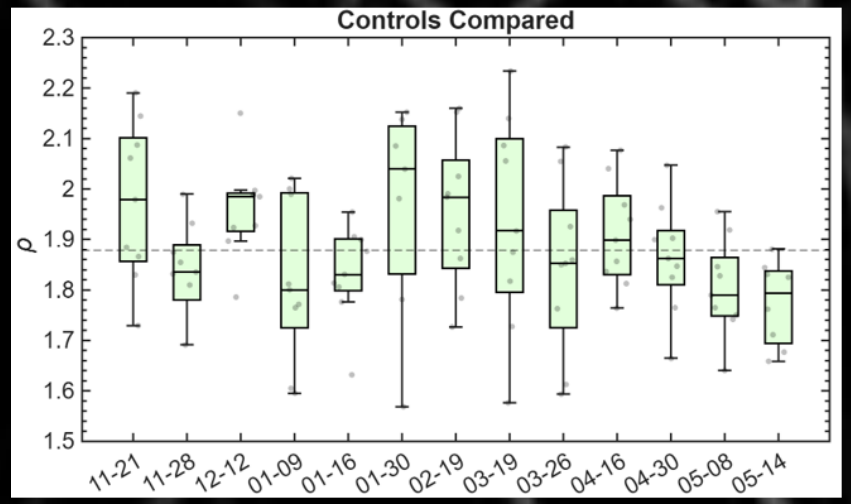
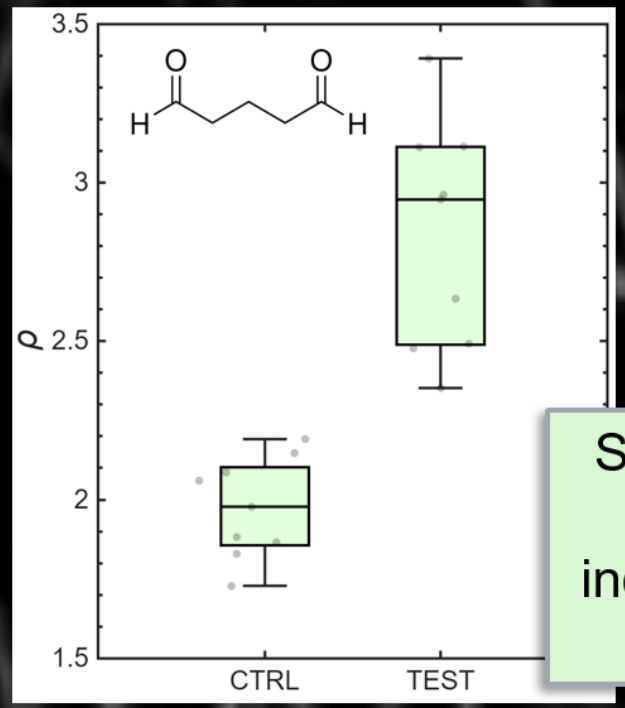
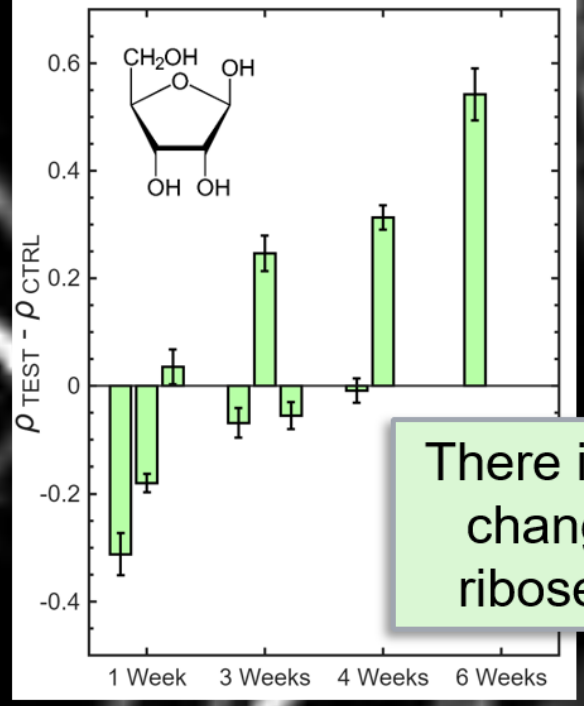


In-vitro model for collagen glycation research

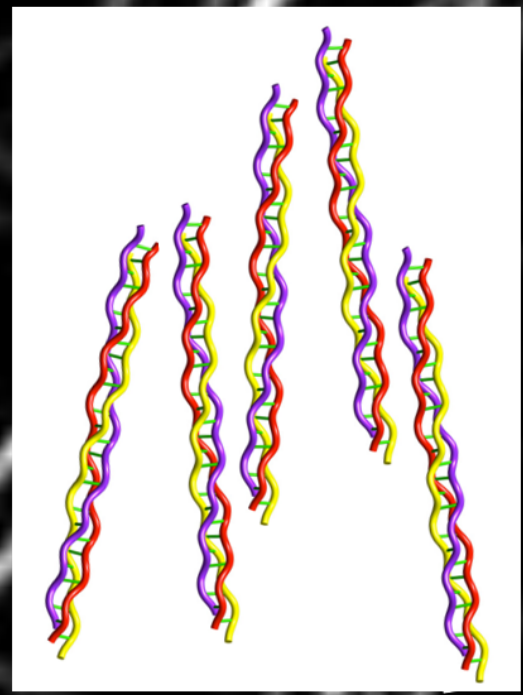


SHG can detect crosslinking-induced changes in collagen

Thank You!

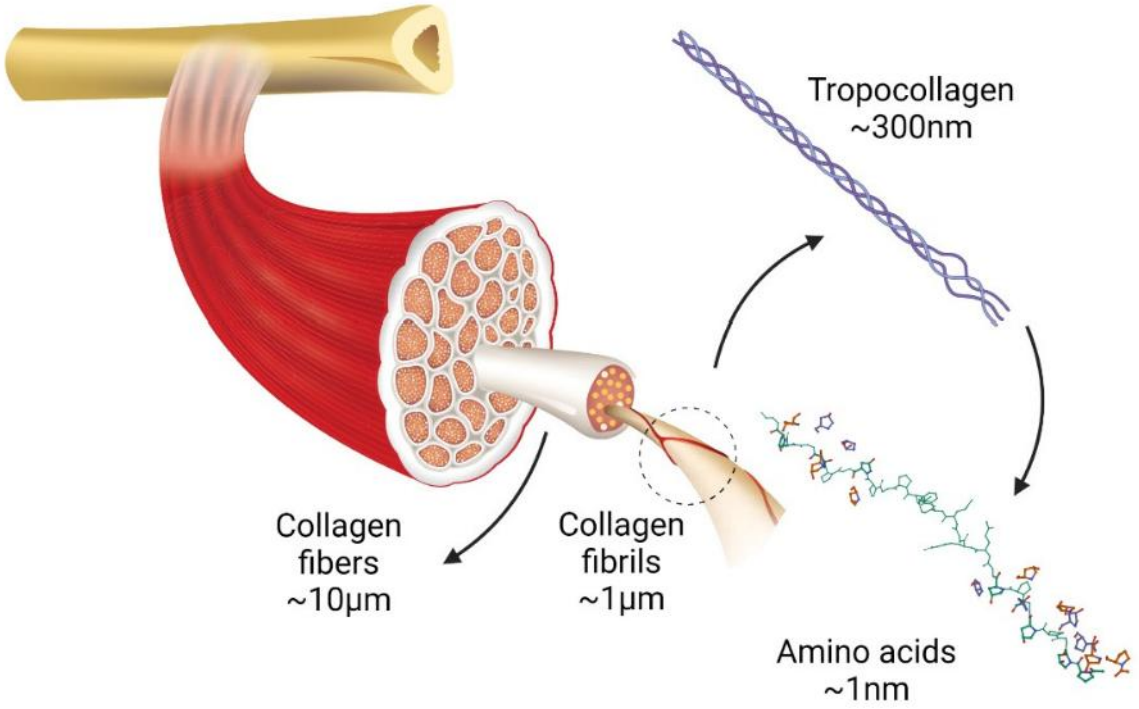


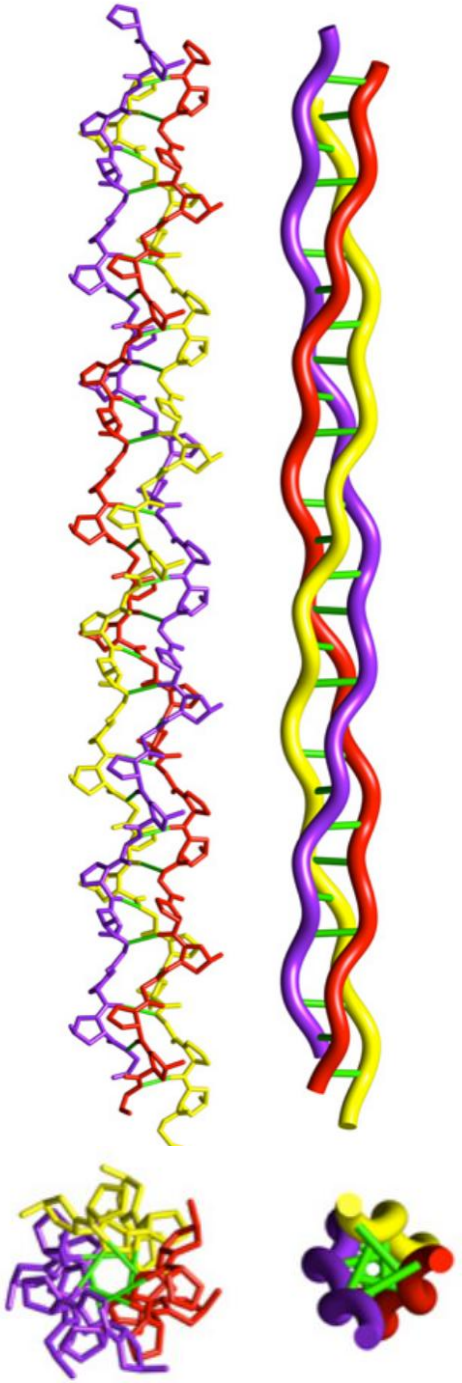
There is a nonlinear change in ρ with ribose incubation



Glycation could cause orientational disorder

Collagen



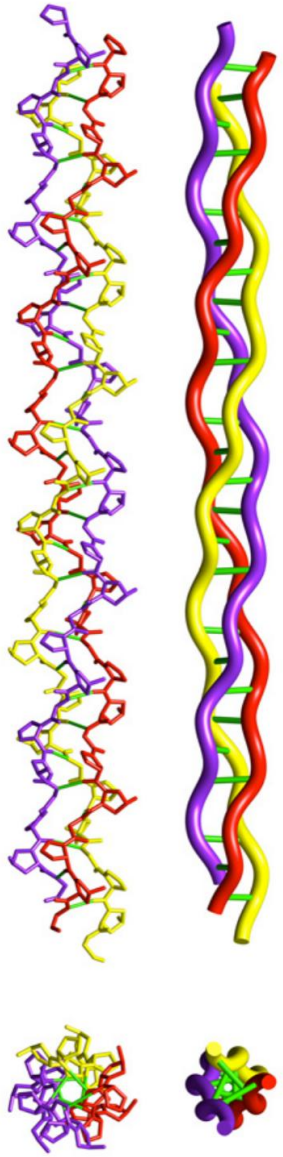


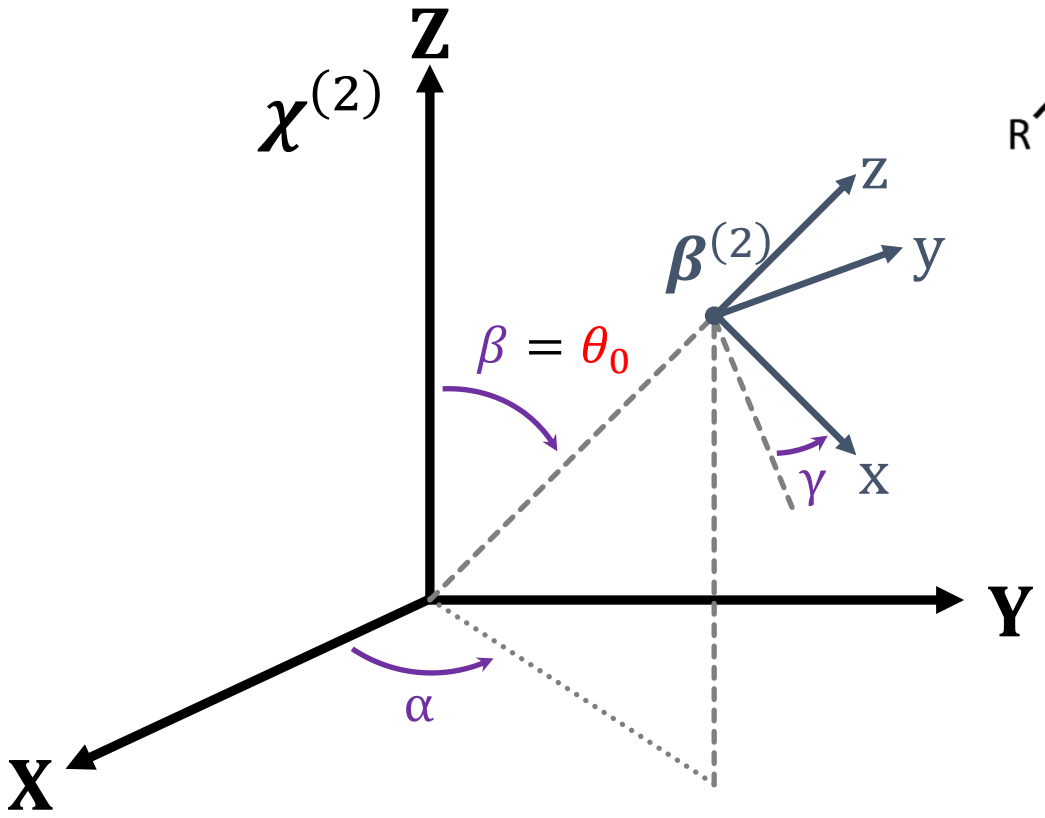
Amirrah, I. N., et. al., Biomedicines (2022)
Jordi Bella, Biochemical Journal, (2016)

The Coordinates of the Collagen SHG Signal

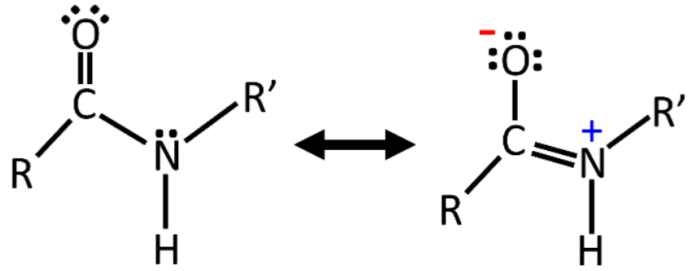
Collagen triple helix structure

$\chi^{(2)}$





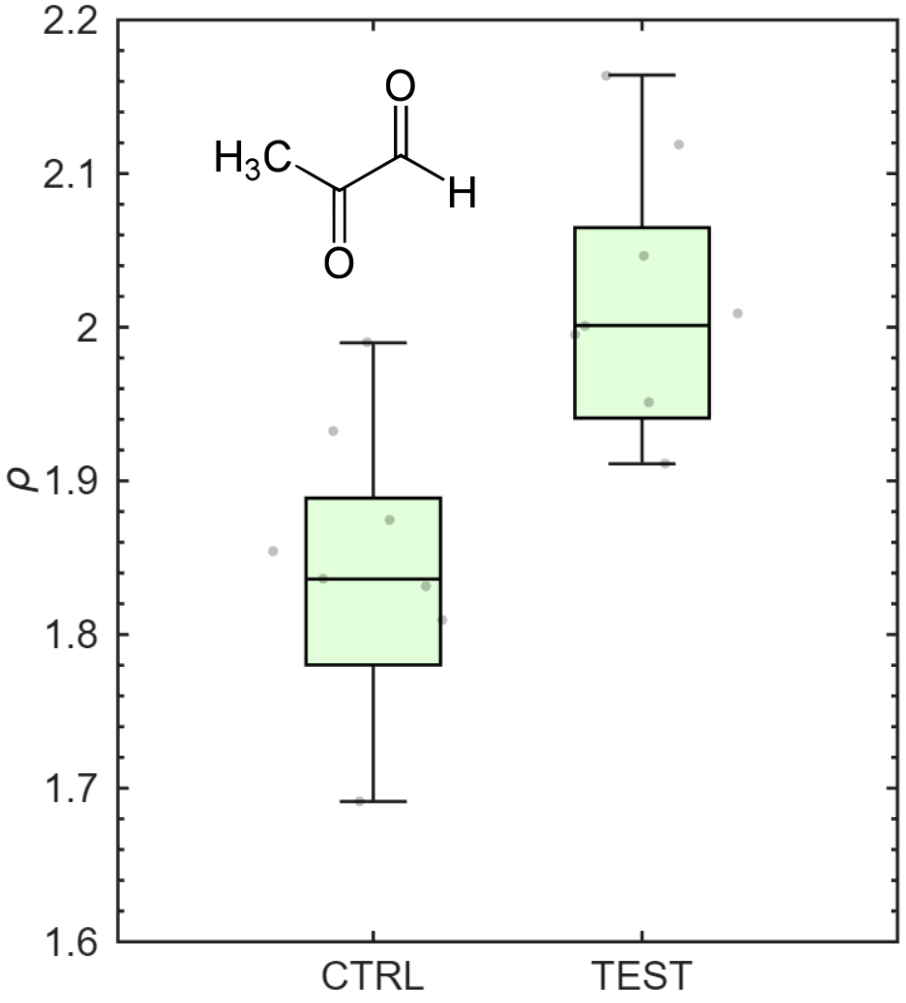
Coordinate system for collagen SHG



Amide bond resonance

$\beta^{(2)}$

Methylglyoxal Replicates Glutaraldehyde Experiment



PBS Control
$n = 9$

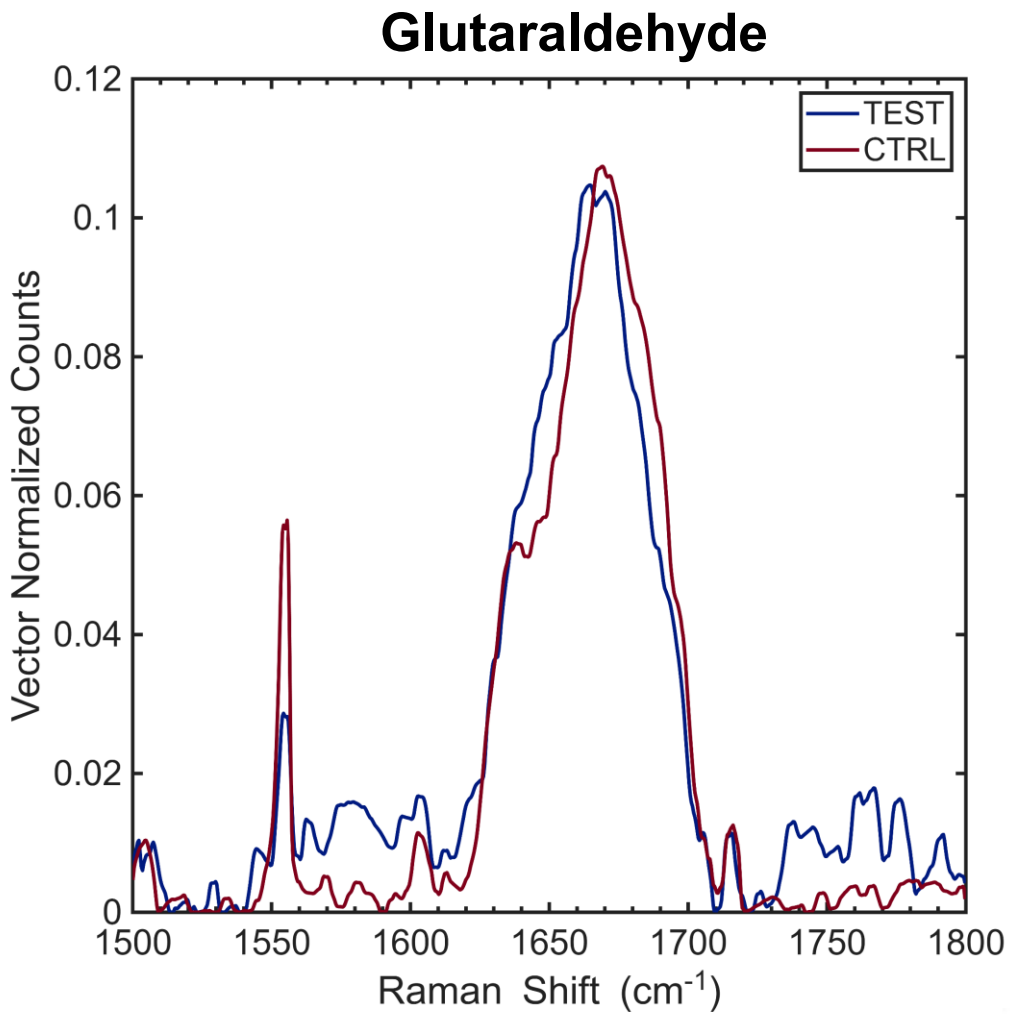
Methylglyoxal
50 mM 24 hours
$n = 9$

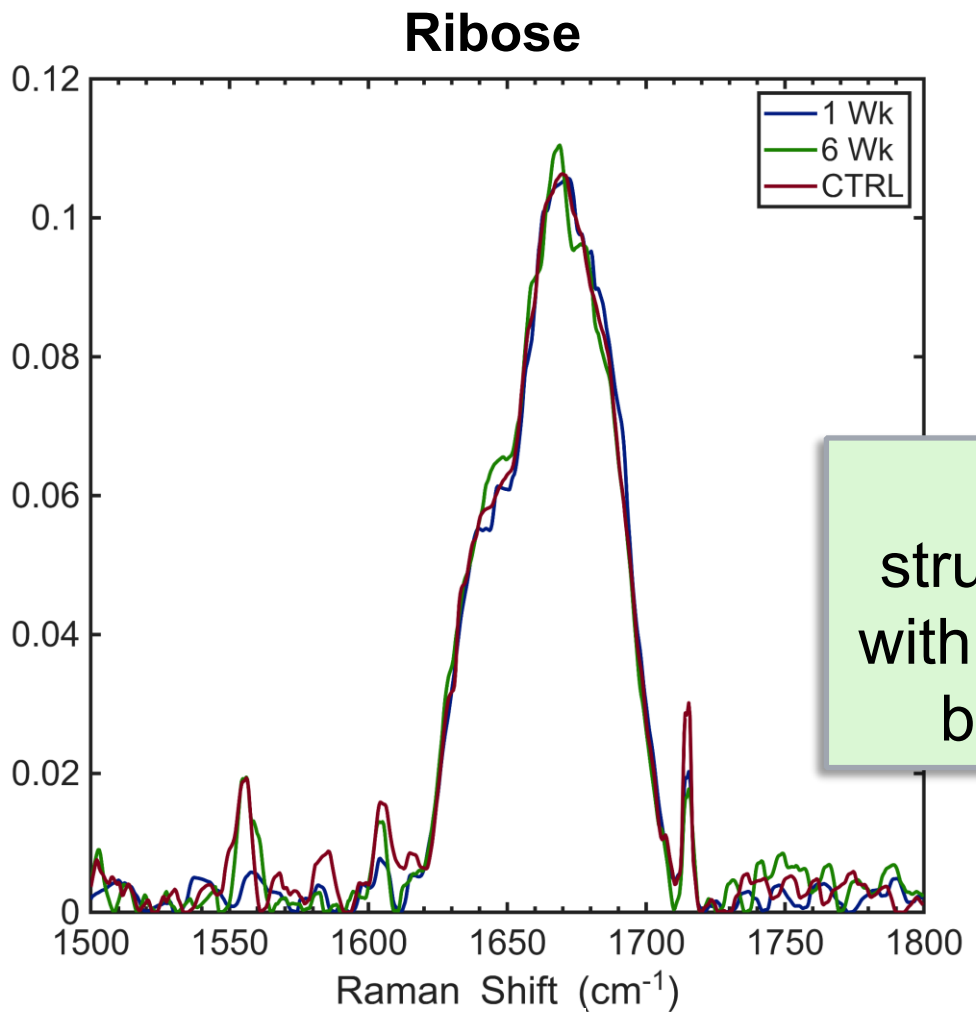
Sample	CTRL ρ	TEST ρ	$\Delta\ \rho$	p (T-Test)
GA	2.03	2.88	0.85	0.000019
MGO	1.81	1.96	0.14	0.059

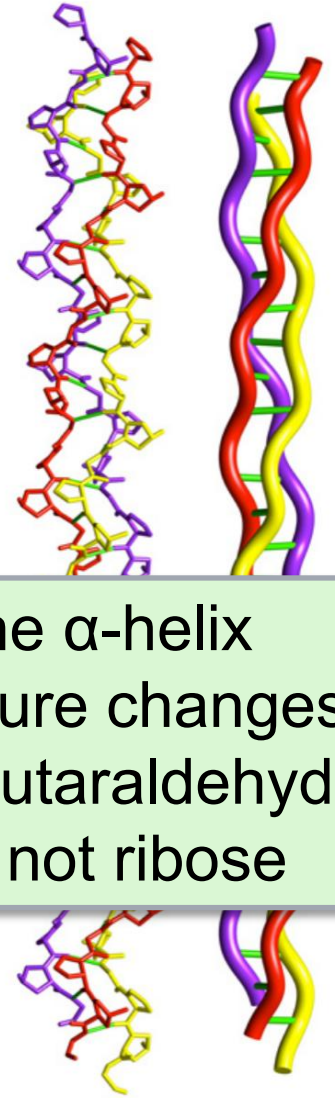
- Both MGO and glutaraldehyde showed an **increase** in ρ
- MGO showed a **smaller** and **less significant** shift than glutaraldehyde

✓ Methylglyoxal repeats the same trend as glutaraldehyde

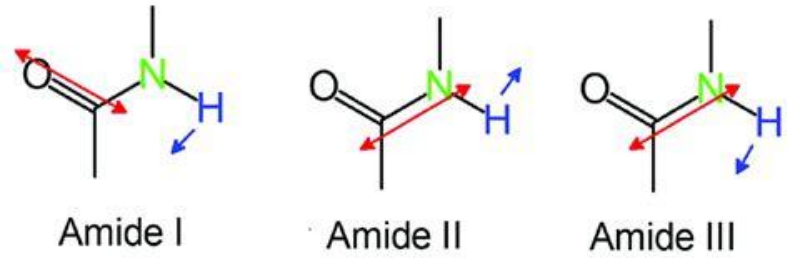
Raman Amide I Band Shows Limited Change



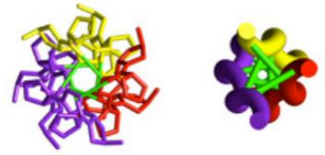




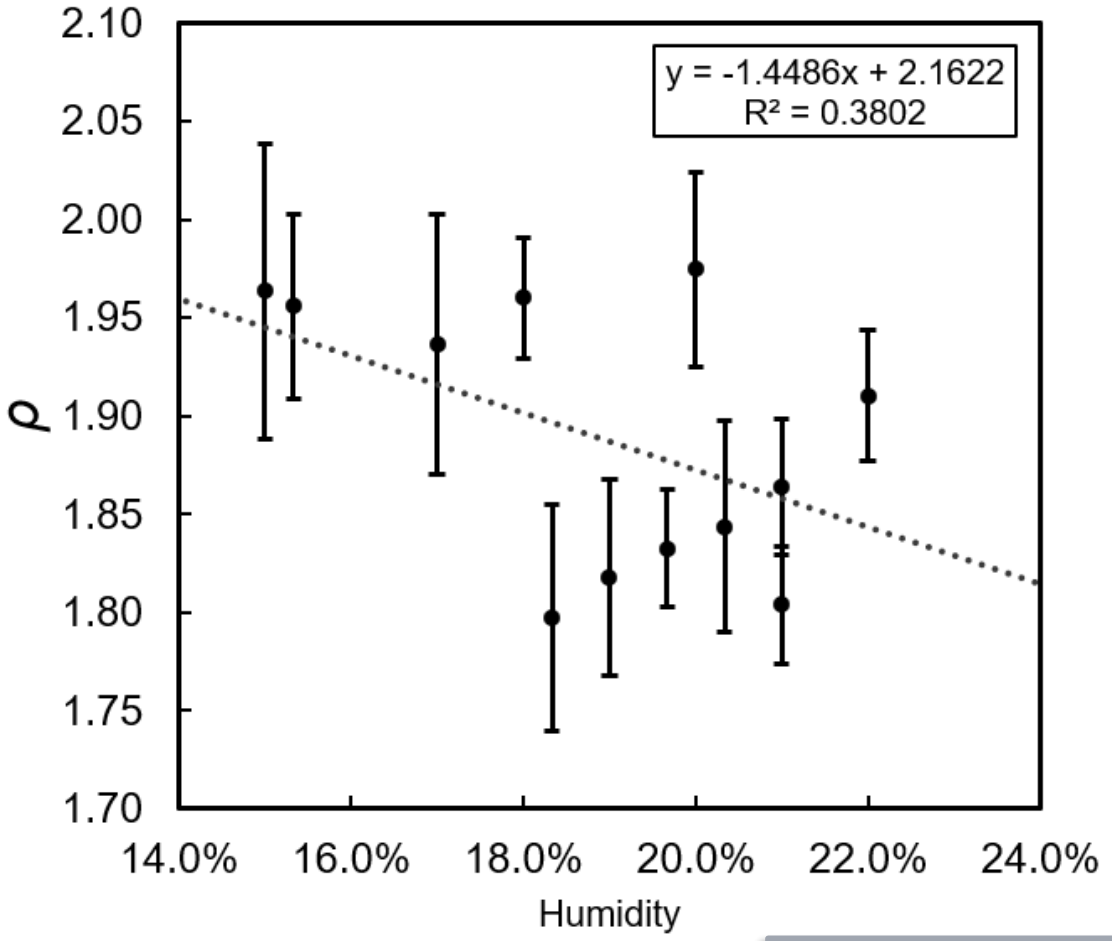
The α-helix structure changes with glutaraldehyde but not ribose

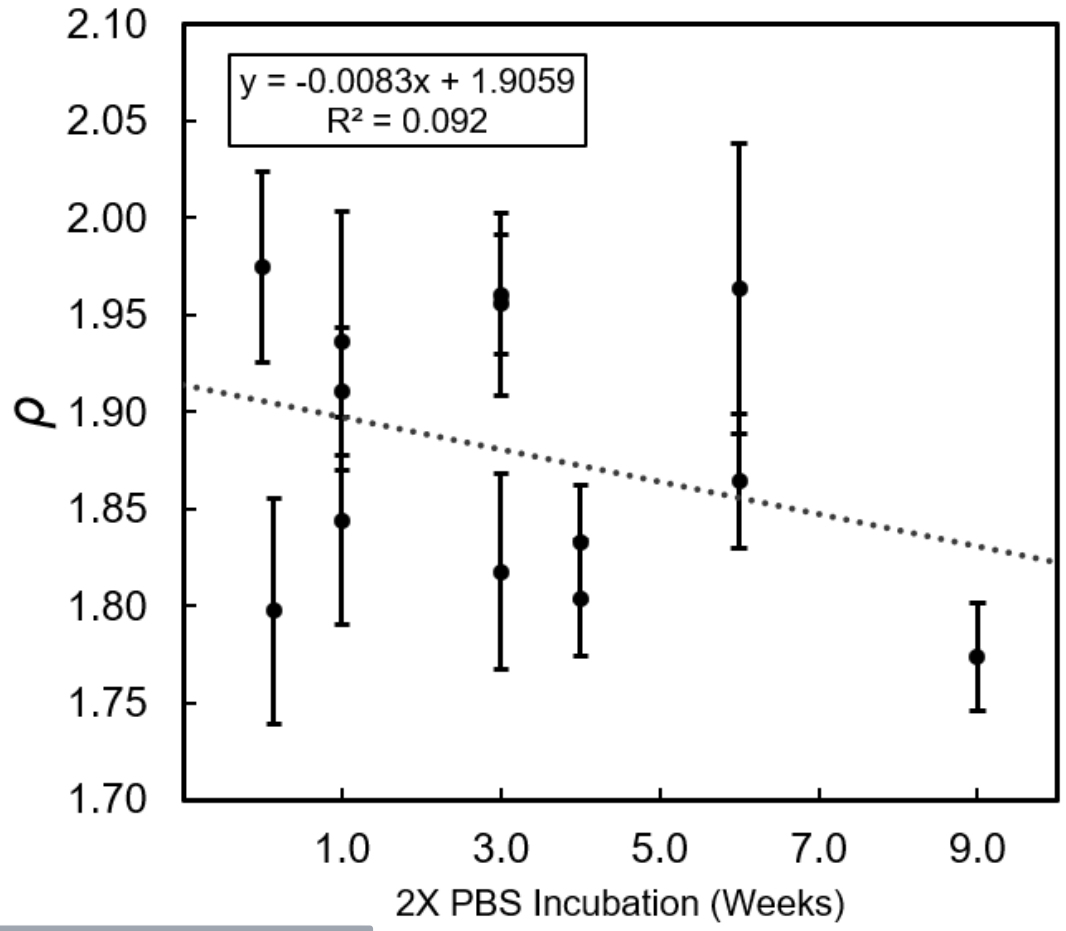


M. Ichikawa, Micro, (2023)



Controls are Not Sensitive to Environment

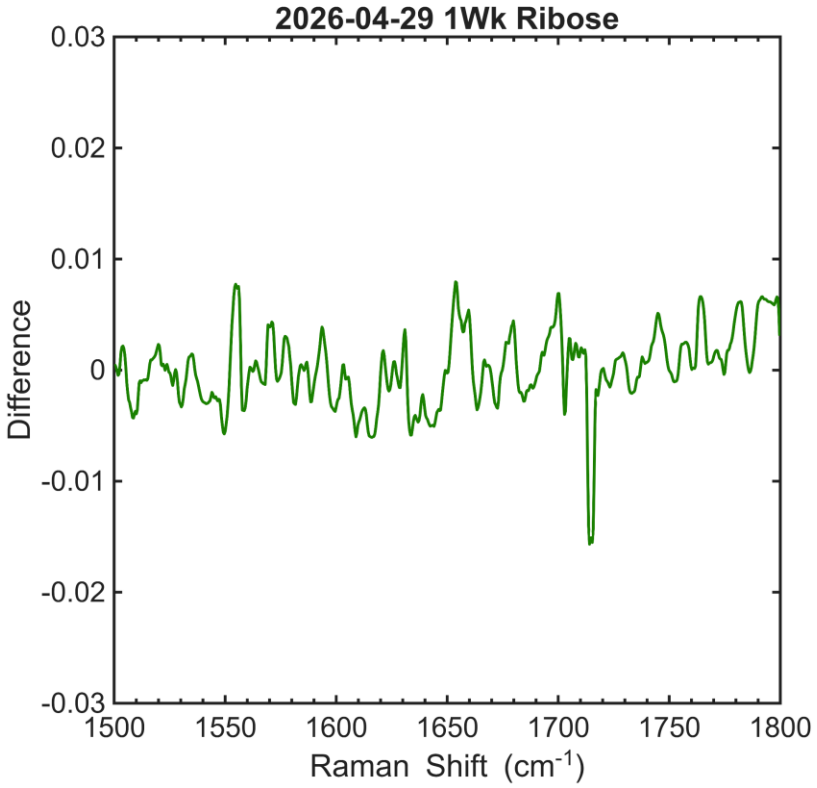


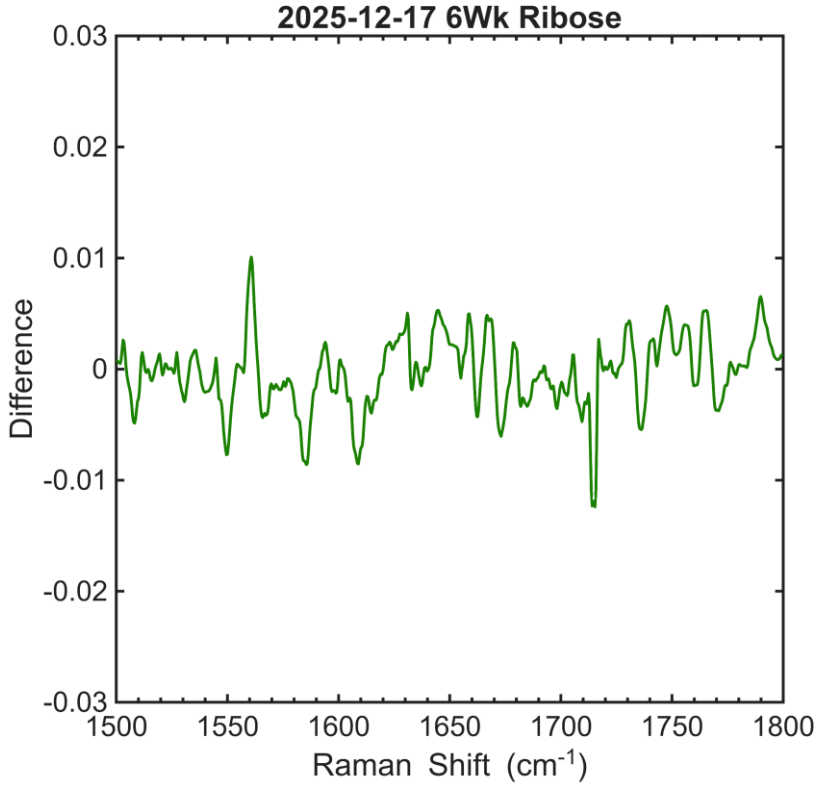


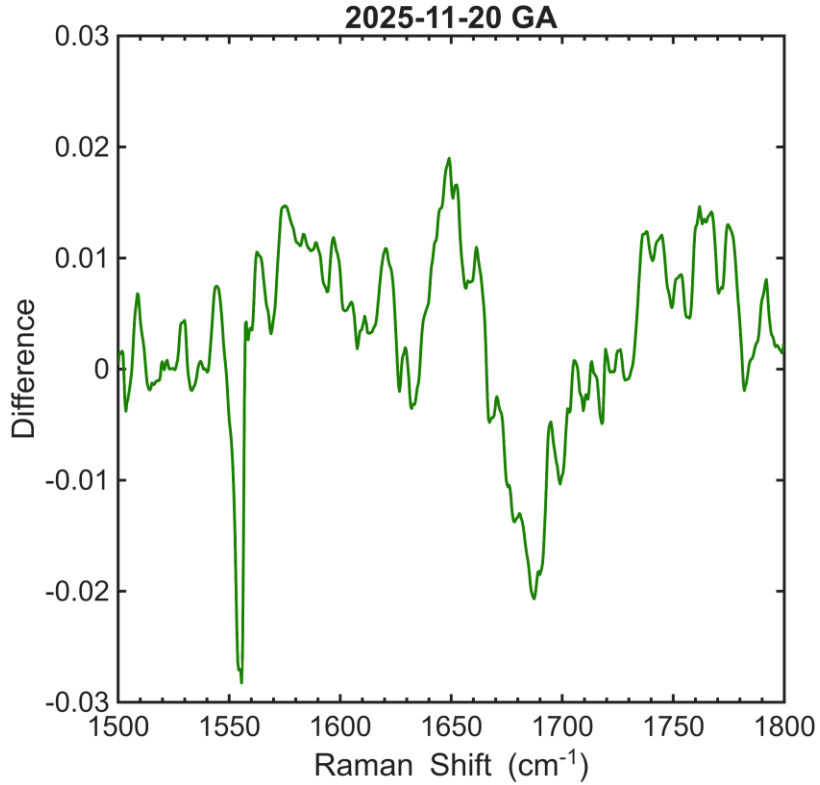
- ✓ Develop a robust, crosslinking-free in-vitro collagen platform


Thank You!

Raman Difference Plots

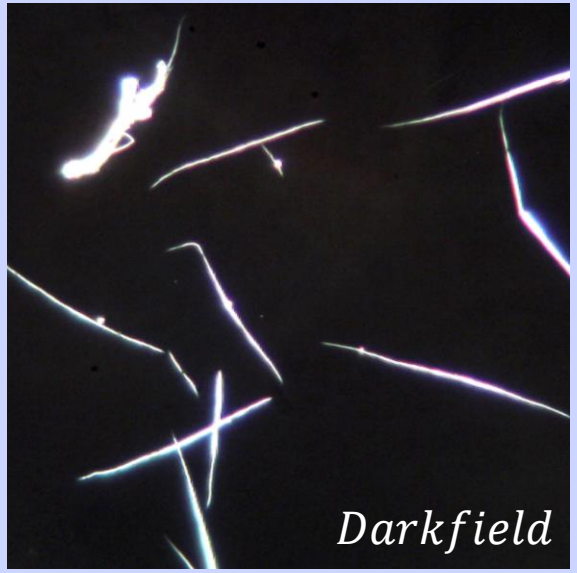


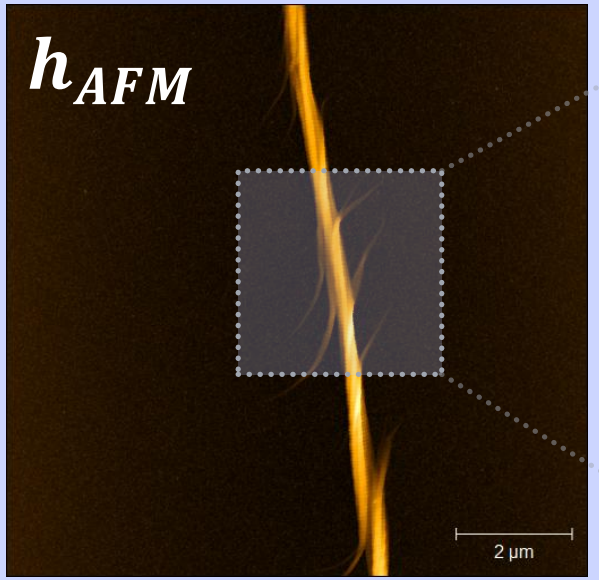


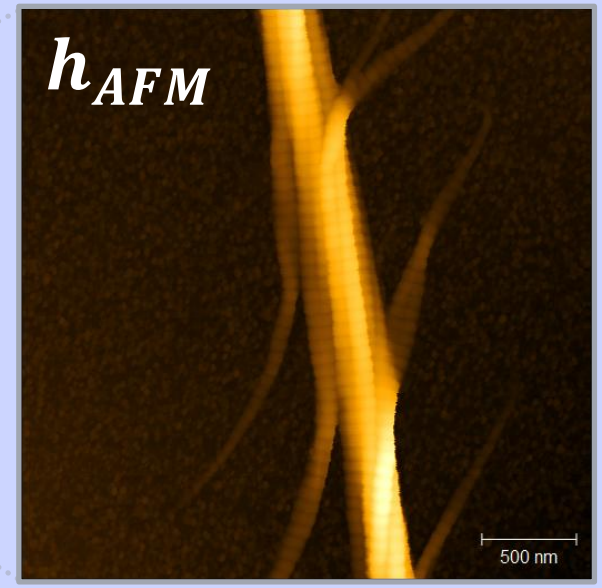


Fraying of Telocollagen Fibrils Increases with Ribose

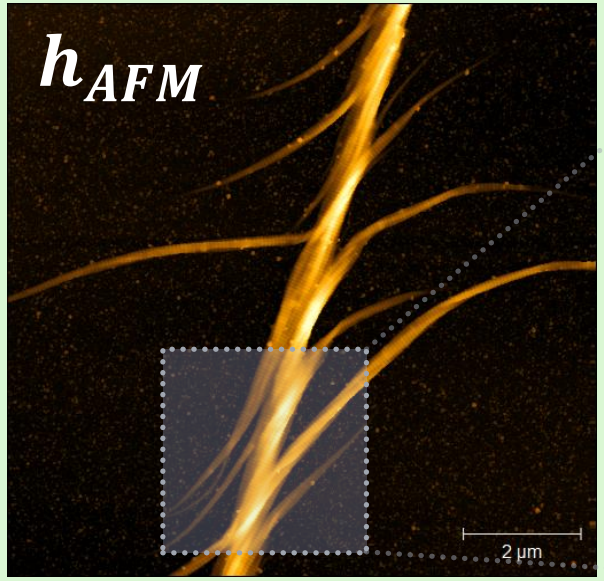
Control

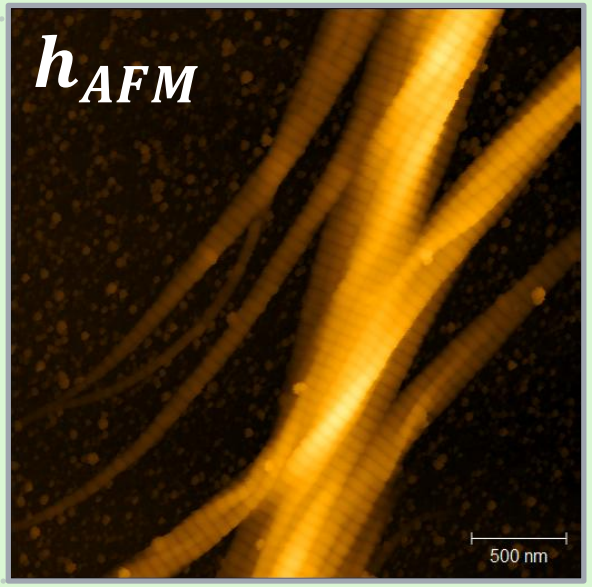






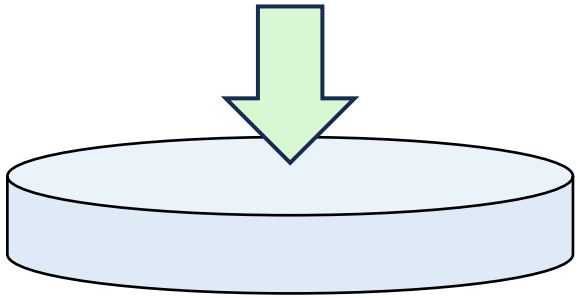
1 Week
0.2 M
Ribose



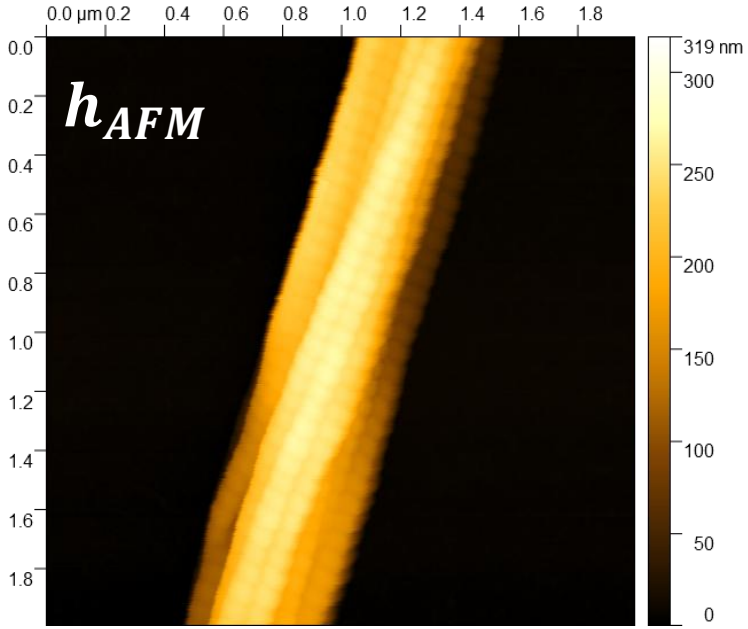


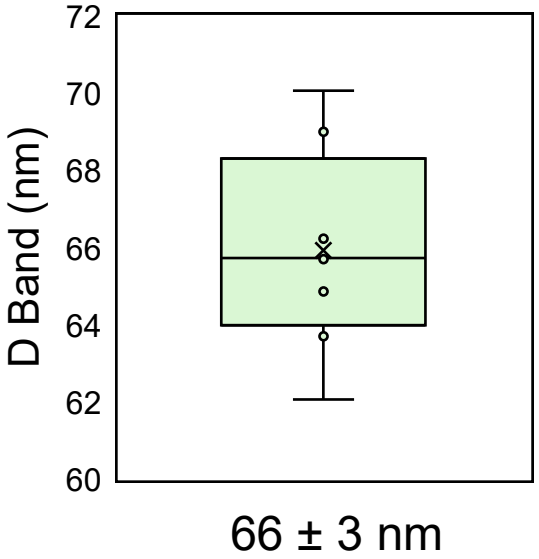
An *In-Vitro* Fibril Platform

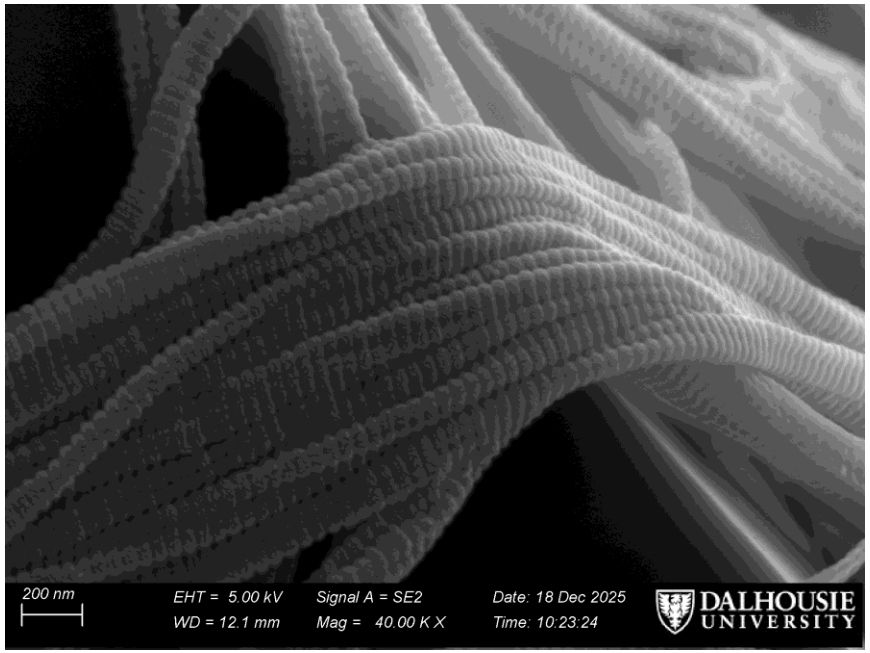
2X PBS
+
Bovine
Telocollagen

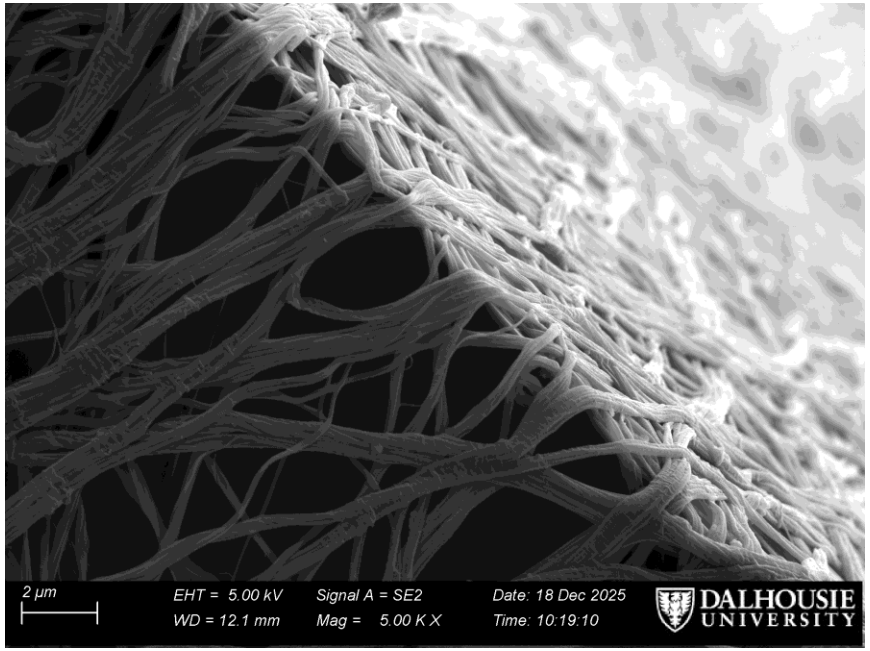


24 hours
incubation
at 30°C

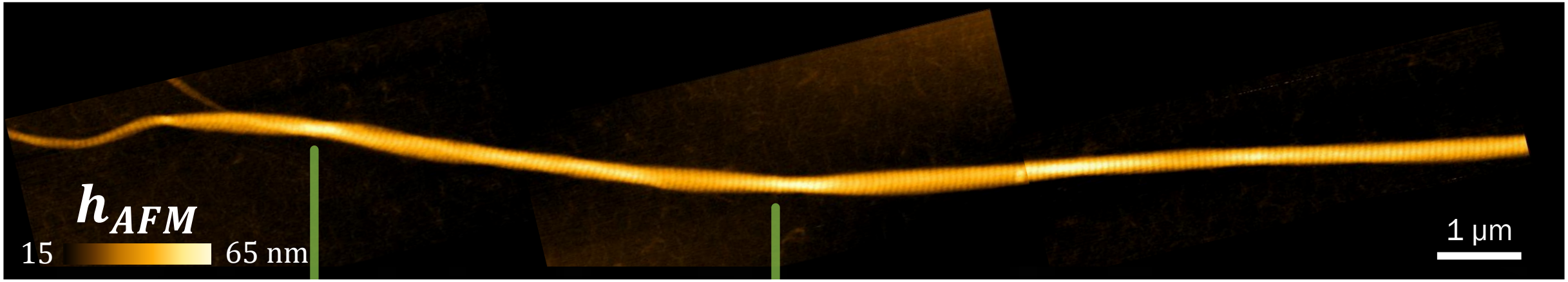


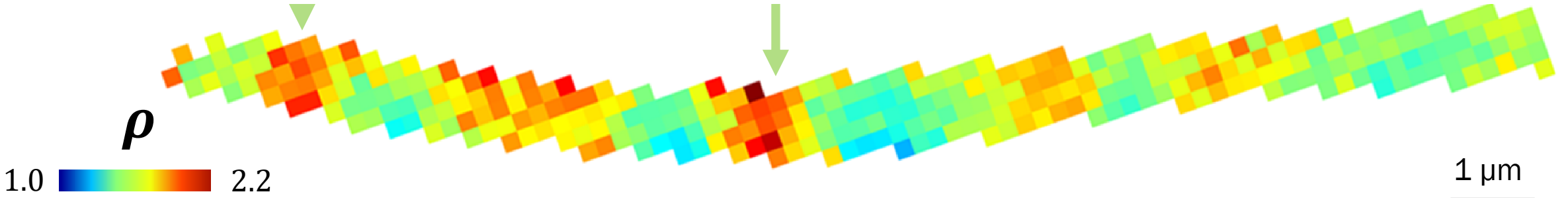




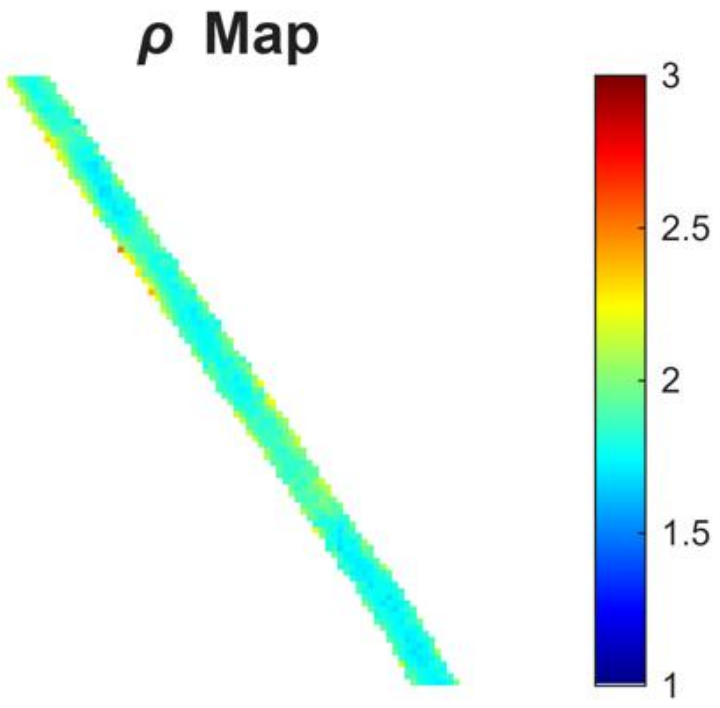


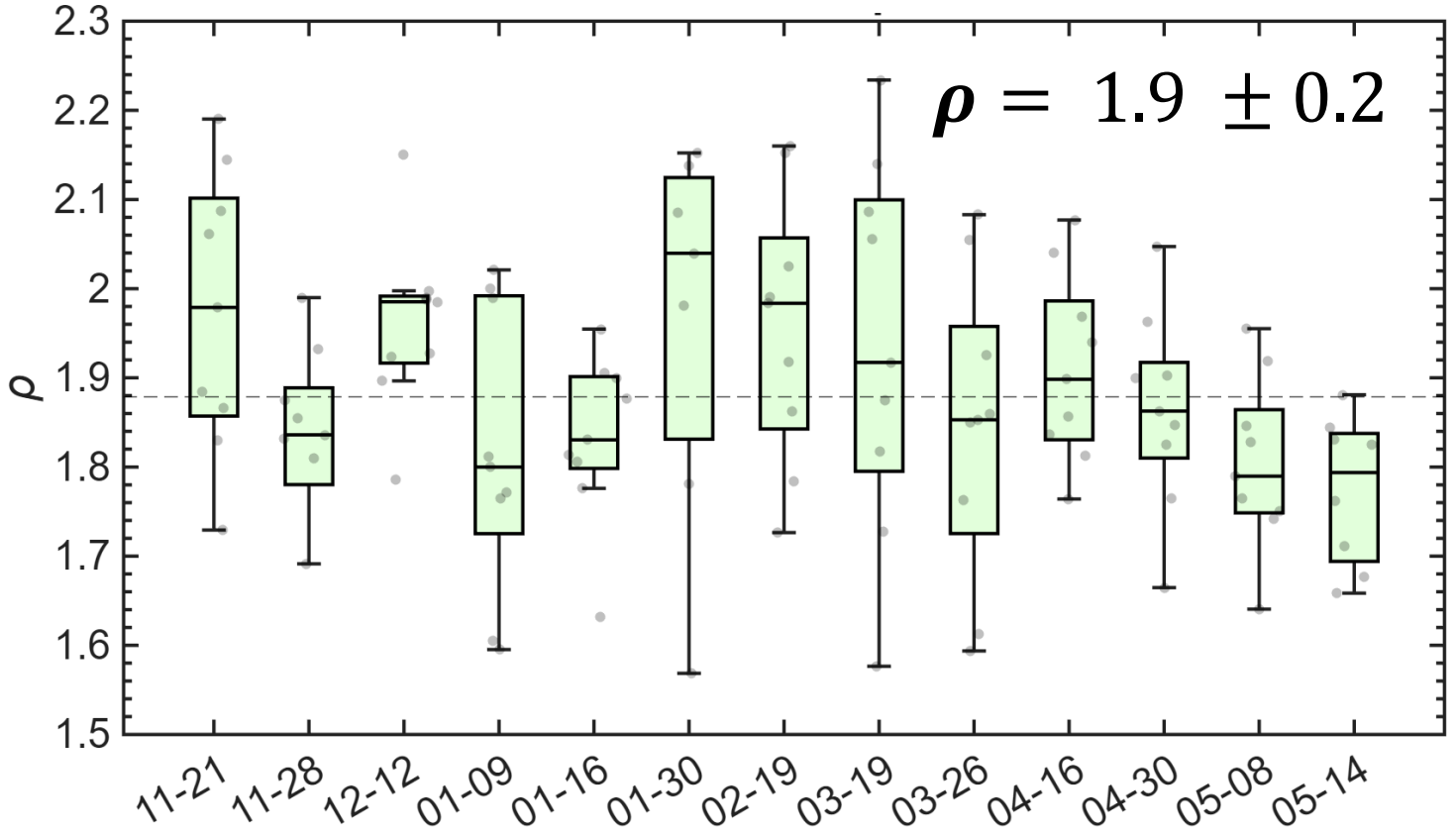
SHG of Collagen Fibrils Reveals Nanoscale Information





Reproducible Controls Across 13 Experiments





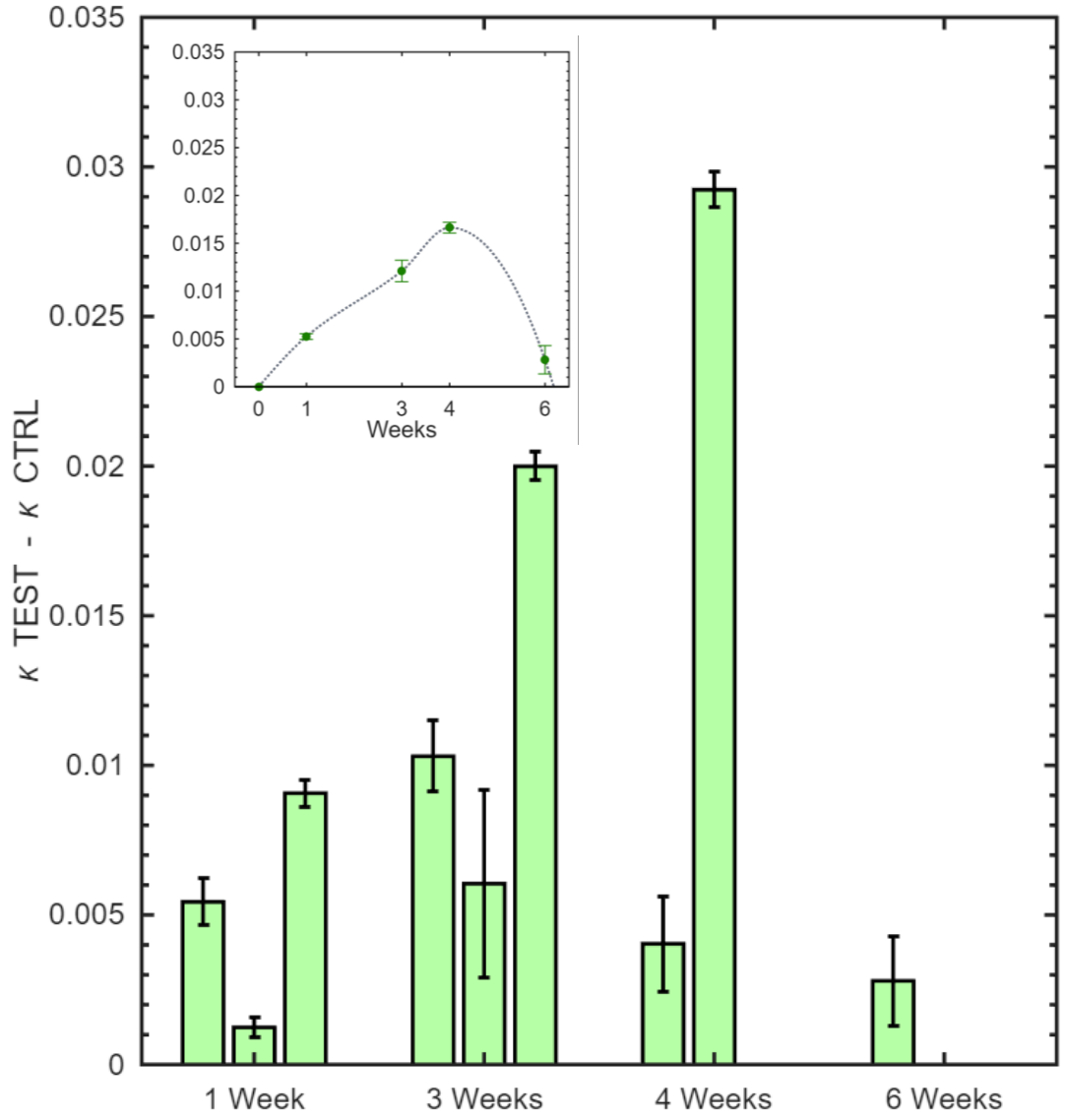
Collagen fibrils tend to have homogenous *ρ*, however, there are fibril-to-fibril differences

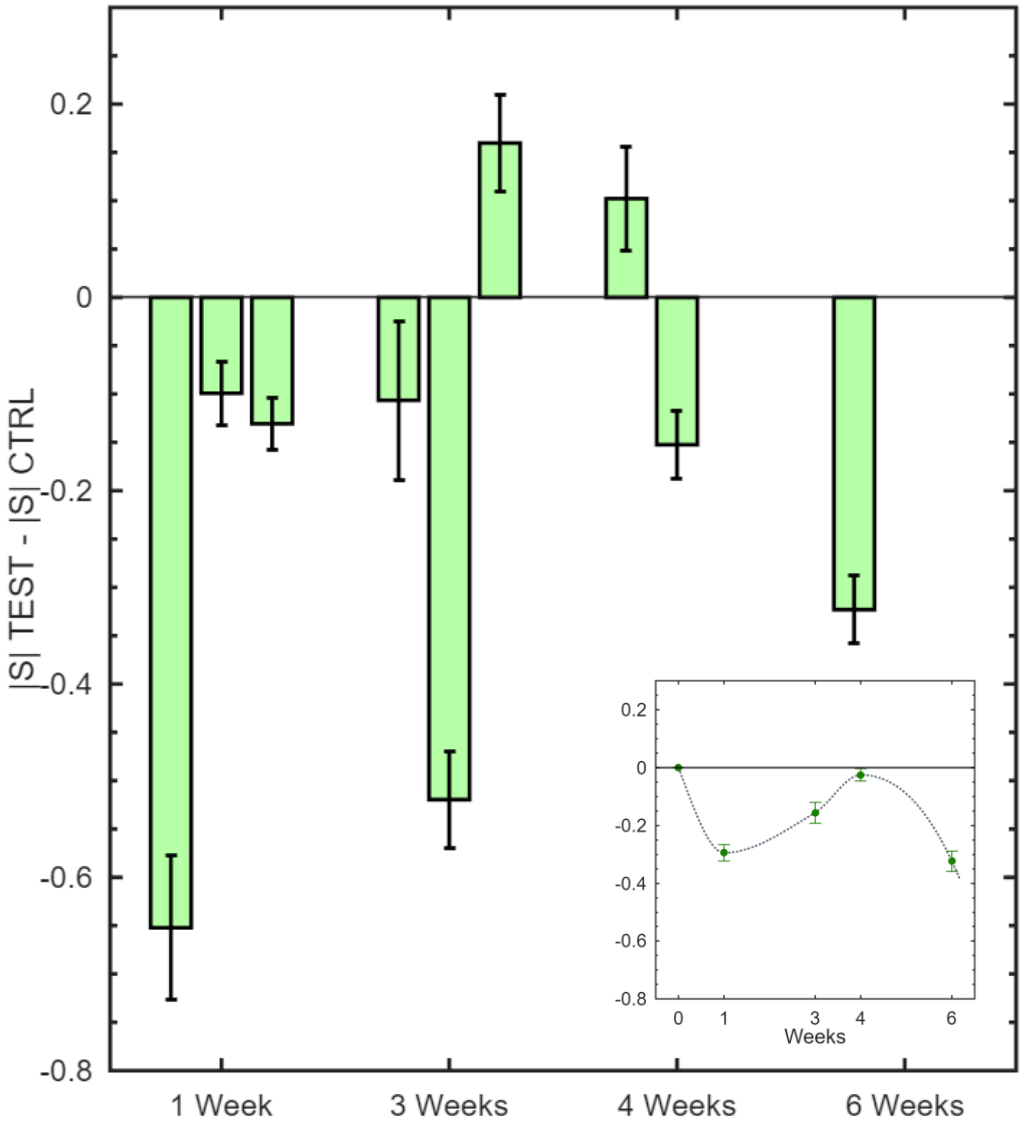
Due to synthesis variations, a new control is acquired for every experiment

$\boldsymbol{n = 114}$ fibrils measured

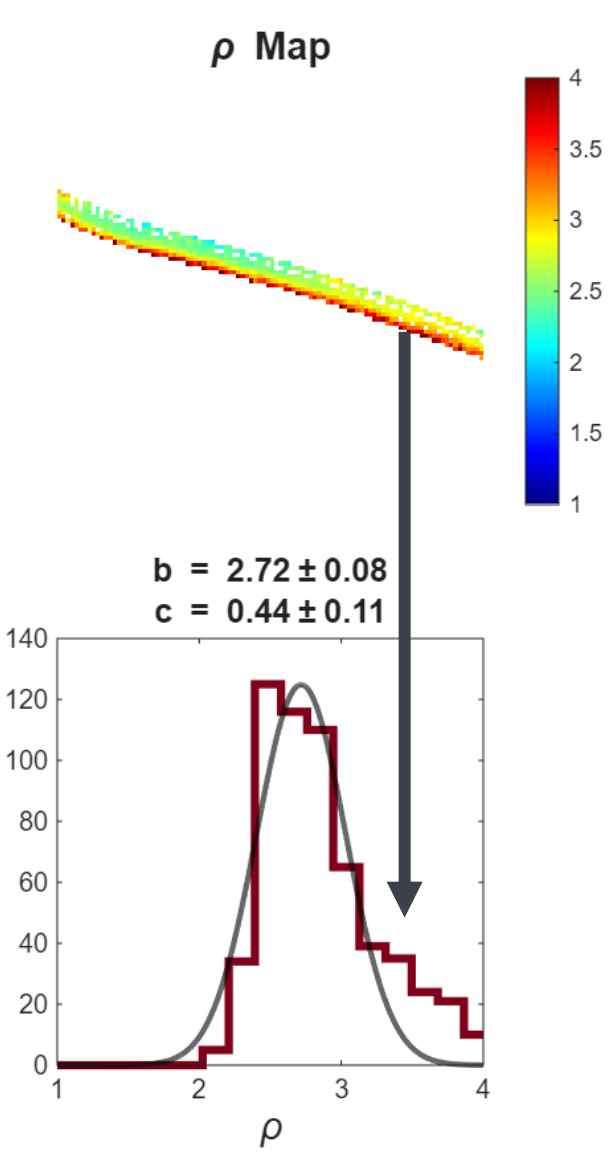
Chiral (κ) and Asymmetry (S) Parameters

$$\kappa = \frac{\chi^{(2)}_{XYZ}}{\chi^{(2)}_{ZXX}} \quad S = \frac{\chi^{(2)}_{XXX}}{\chi^{(2)}_{ZXX}}$$

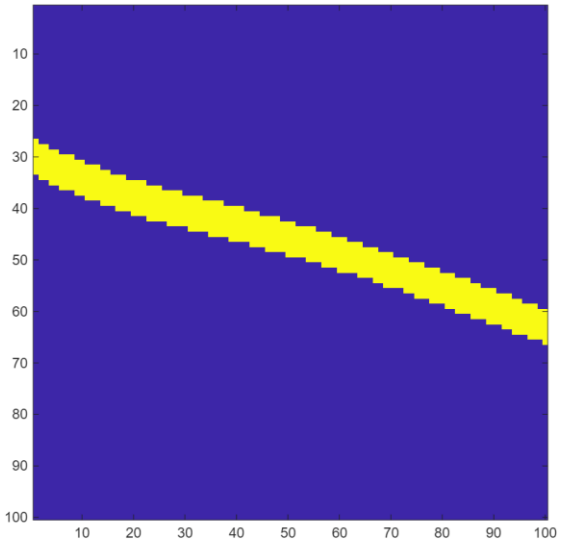




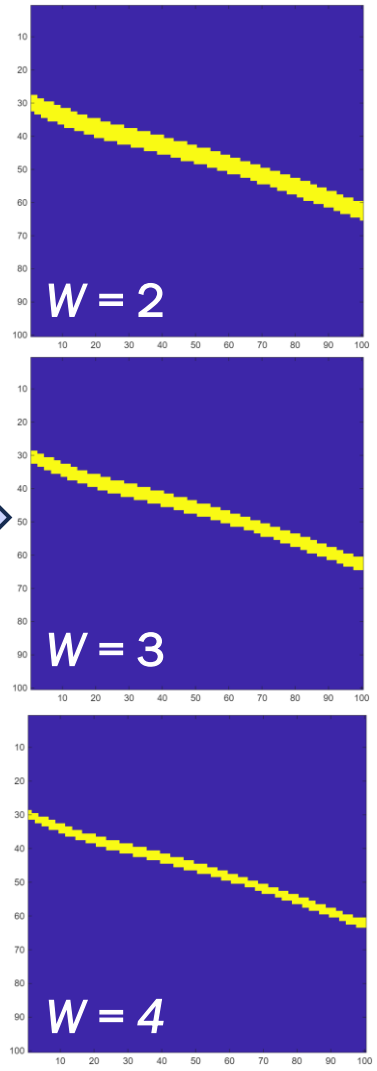
Removing Outlier Pixels with Erosion Filtering



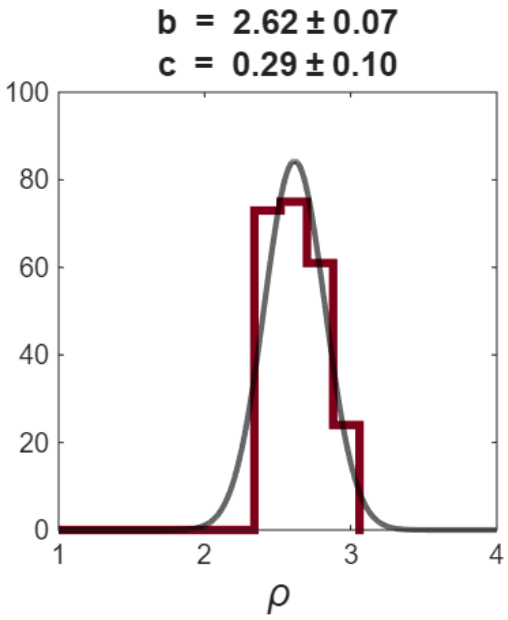
Binarized Image
With Morphological Closing
Square Width $W = 3$



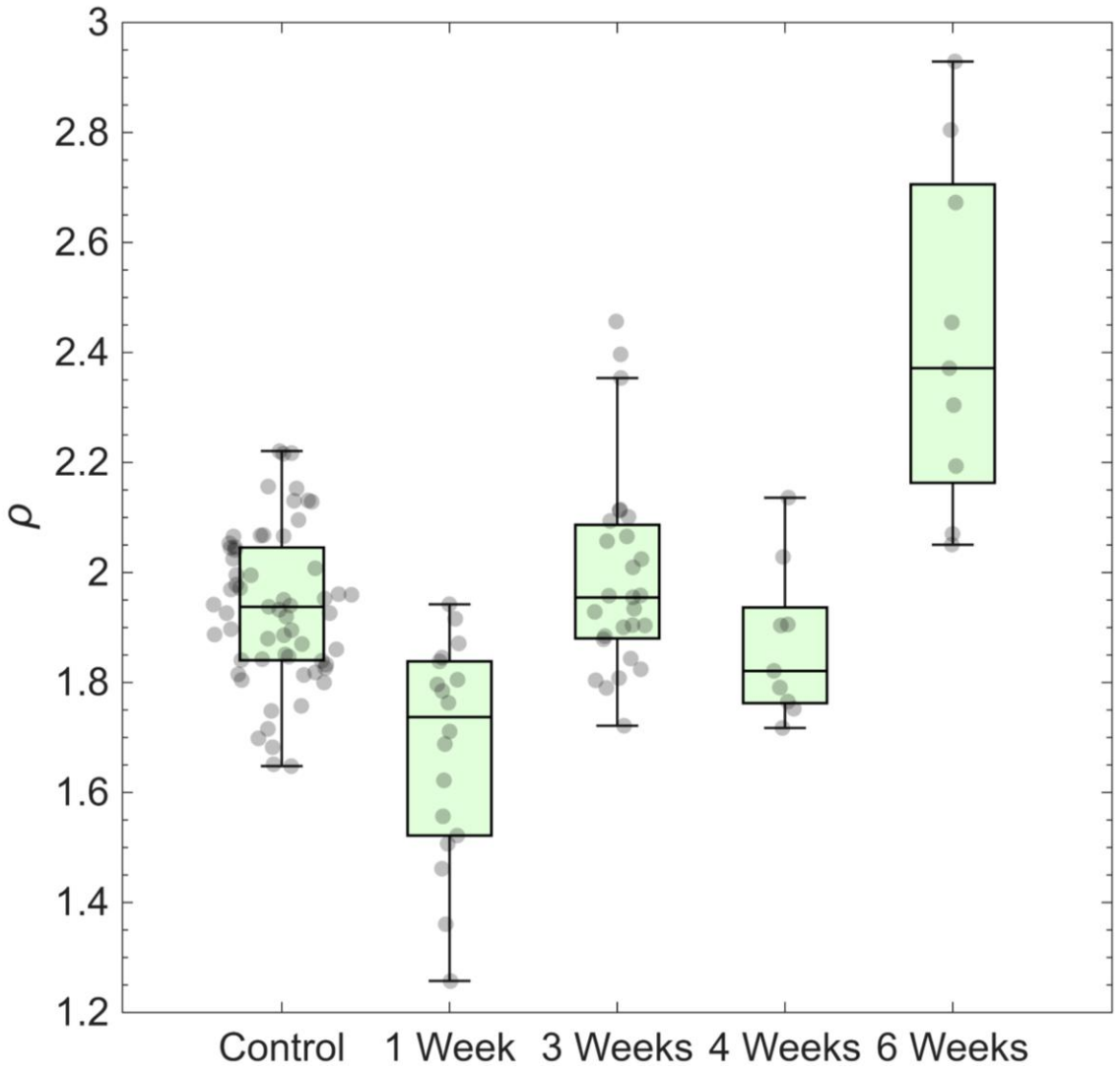
Eroded Images

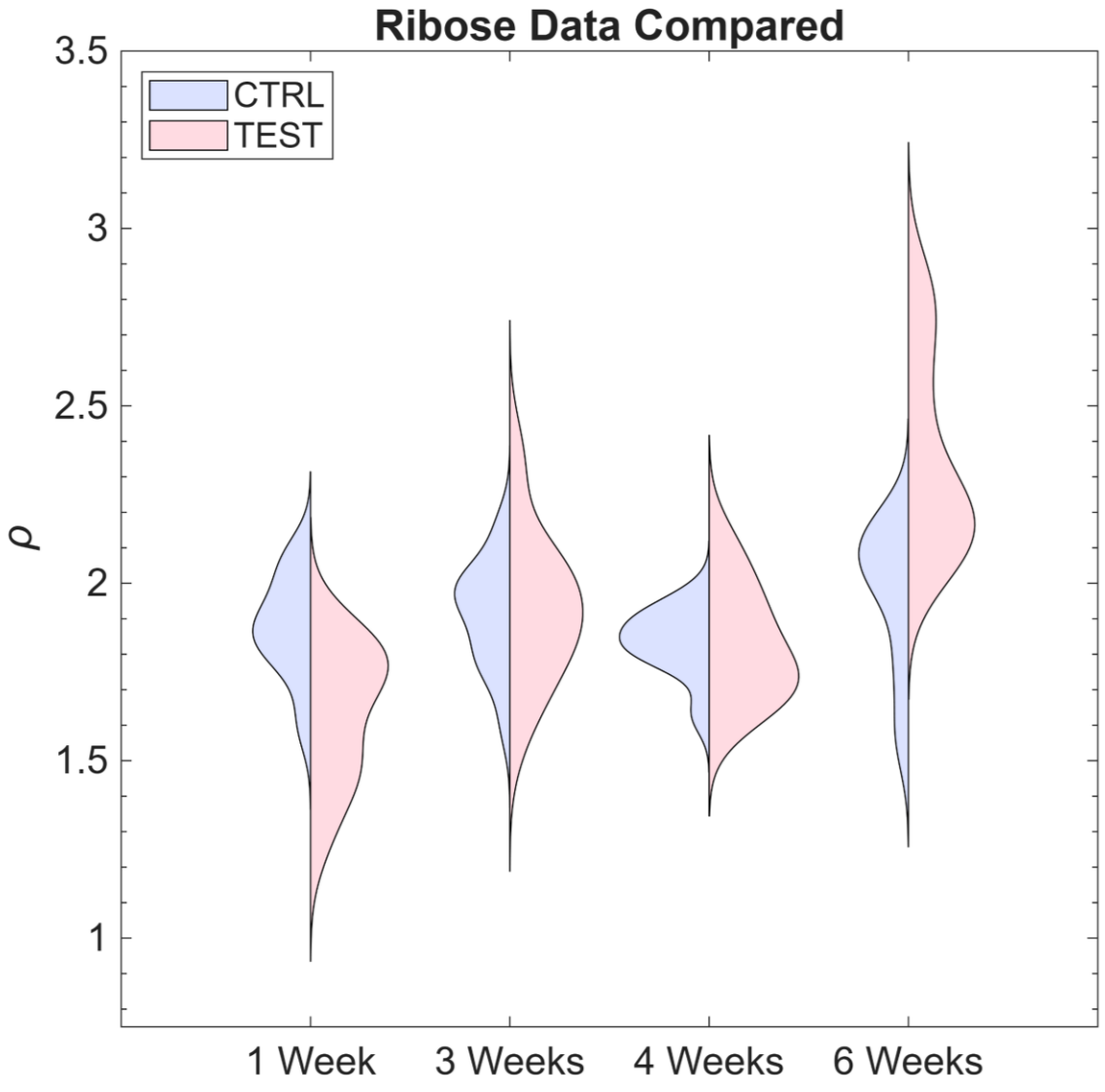


Square Erosion W = 4



Alternative Ribose Plots





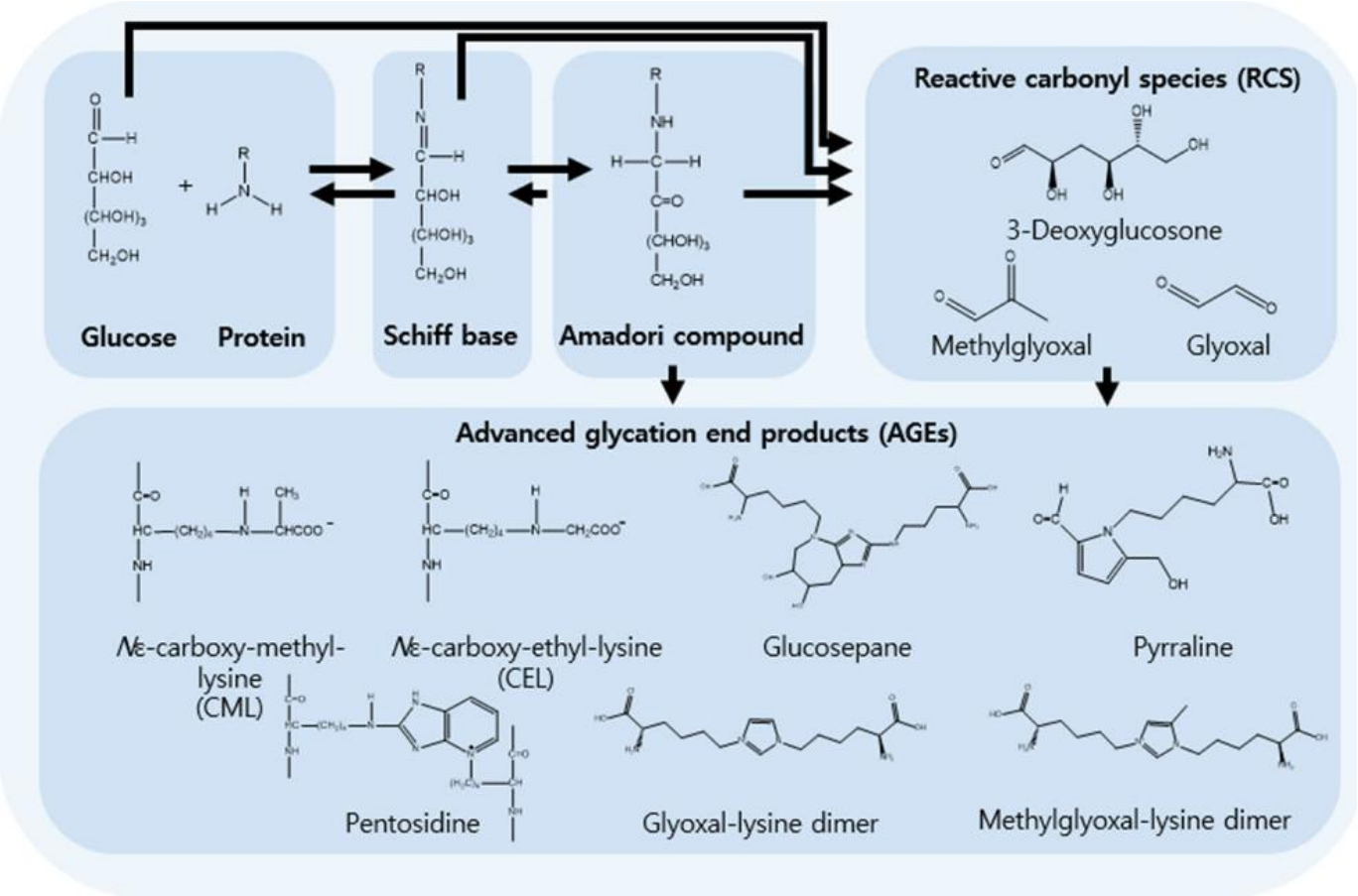
Conclusions

- We developed a robust *in-vitro* model for collagen SHG
- SHG is highly sensitive to crosslinking in collagen fibrils
- Molecular disorder increases in glycated collagen fibrils
- Ribose and glutaraldehyde affect collagen structure differently
- The glycation process is rich, multifaced, and nonlinear

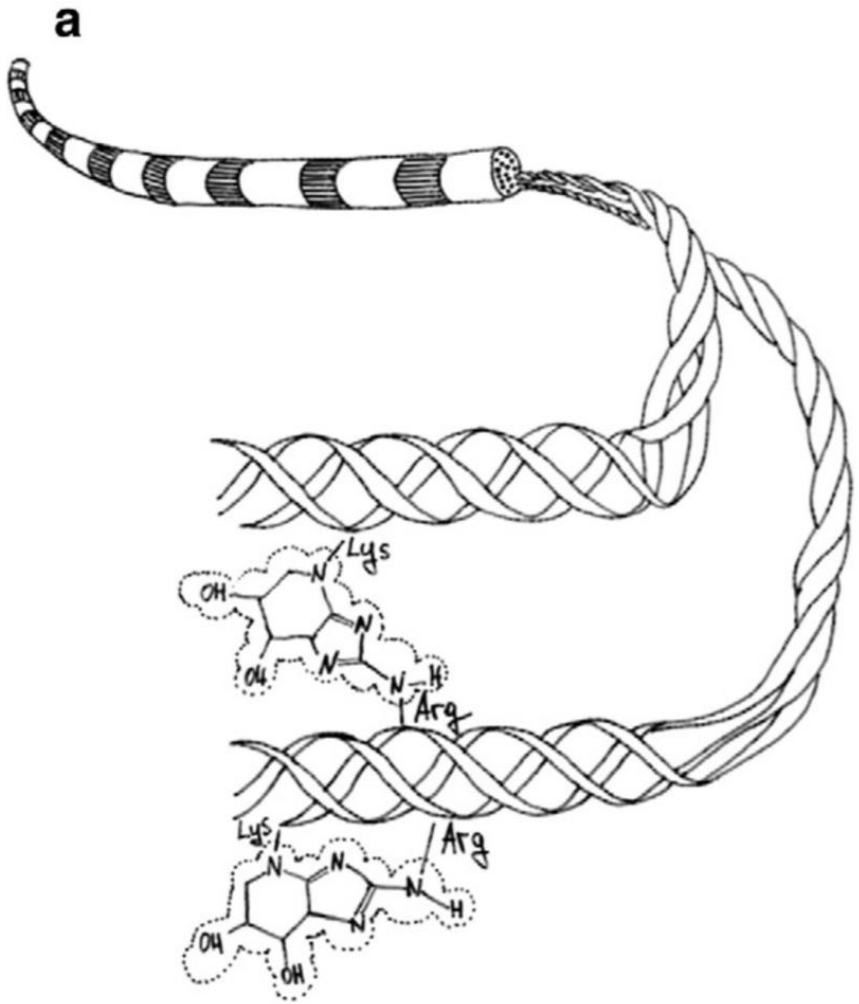
Future Directions

- Additional ribose experiments
- Further glutaraldehyde experiments to distinguish between *intra-* and *inter-* molecular crosslinking
- Additional measurements with fluorescence anisotropy and X-ray diffraction to test the disorder hypothesis
- Exploring further SHG and polarization parameters

Glycation Chemistry of Collagen Fibrils



S. Cho et. al., Reviews in Analytical Chemistry, (2022).



A. Gautieri et. al., Matrix Biology, (2014)

Summary – What Did We Observe?

Glutaraldehyde

1 experiment with 50 mM, 1 hr

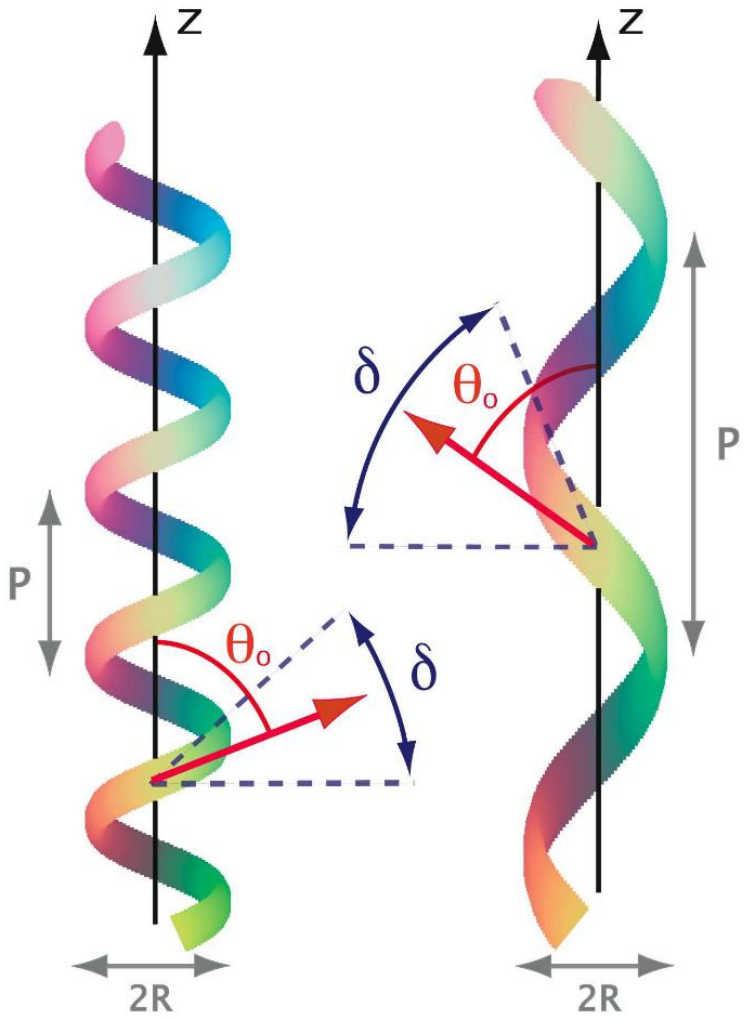
- Pitch angle **decreases** to 40°
- Width of angles **increases**
- D-band remains the **same**

Ribose Time Series

7 experiments with 0.2 M and 1, 3, 4, and 6 weeks

- Pitch angle **decreases** to 42 – 44°
- Width of angles may **increase** at 6 weeks ribose
- Nonlinear trend, **increase** in pitch at 1 week
- D-band **decreases** with incubation time

Using PIPO-SHG to Find Helical Pitch Angle



Tiaho et. al., Optics Express, (2007)

$$\rho = \frac{\chi^{(2)}_{ZZZ}}{\chi^{(2)}_{ZXX}} \qquad \cos^2 \theta_0 = \frac{\rho}{\rho + 2}$$

$$\rho = 3.0 \rightarrow \langle \theta_0 \rangle = 39.2^\circ$$

$$\rho = 2.0 \rightarrow \langle \theta_0 \rangle = 45.0^\circ$$

$$\sigma_\rho \sim 0.05 \rightarrow \sigma_{\langle \theta_0 \rangle} < 1^\circ$$

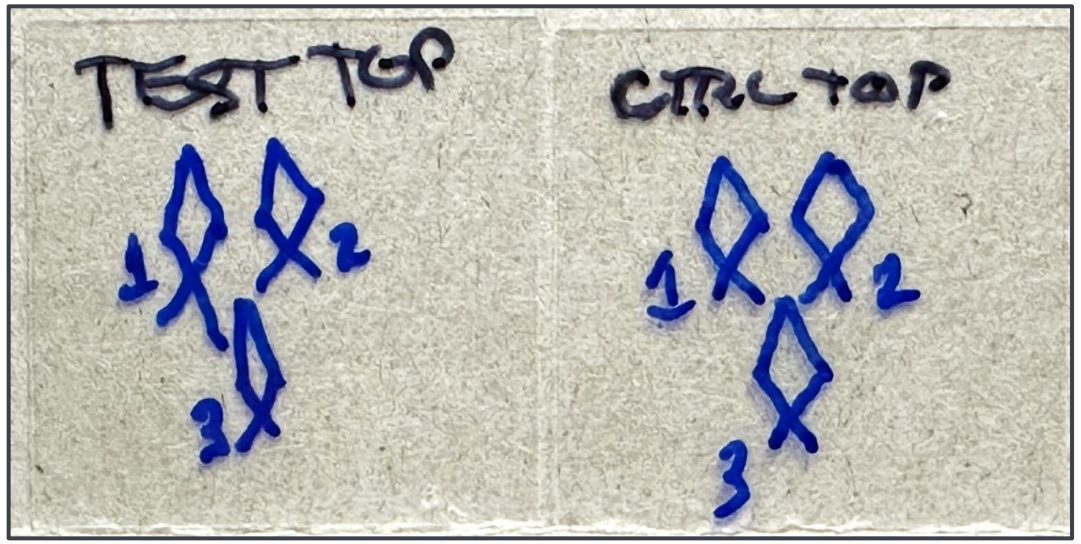
PIPO-SHG can measure pitch angle with sub-degree precision

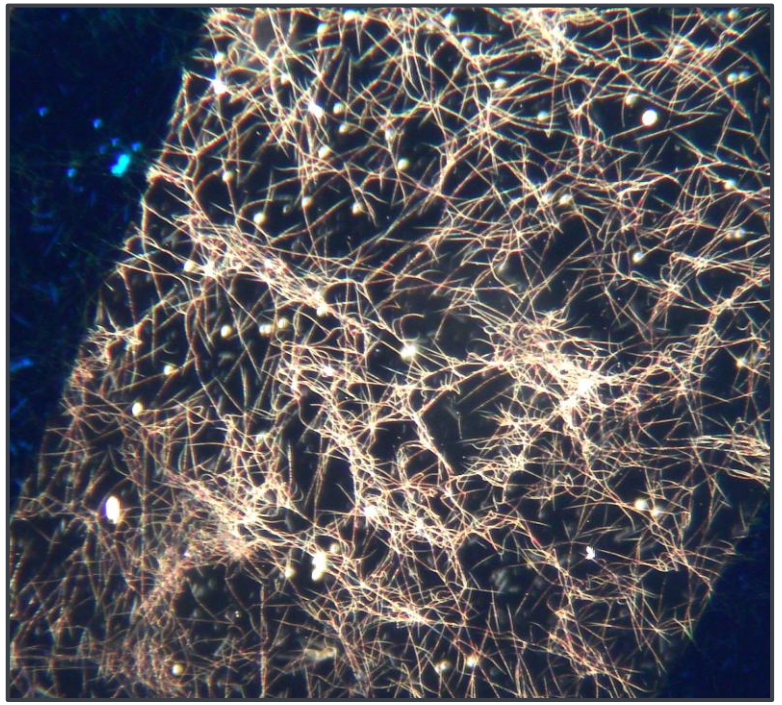
PIPO-SHG Details

For one experiment:

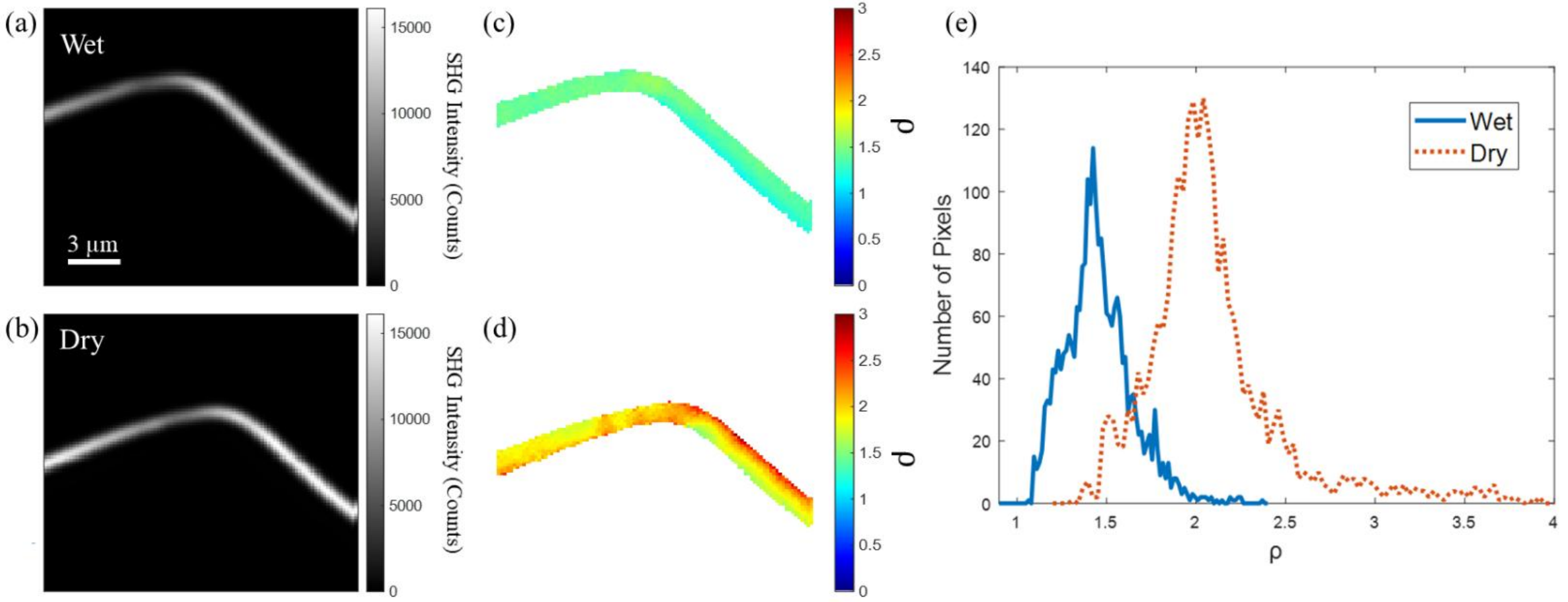
- 9 TEST images
- 9 CTRL images

- 19×19 μm^2 image, 100×100 pixels
- 25 frames per image in the stack
- Laser power adjusted to maximize counts
- Fit afterwards to extract ρ and angle





Typical Ranges for ρ in Collagen Fibrils



Harvey et. al., Nanophotonics, (2023)