Quantitative Imaging of Collagen Fibers Using Second-Harmonic Generation

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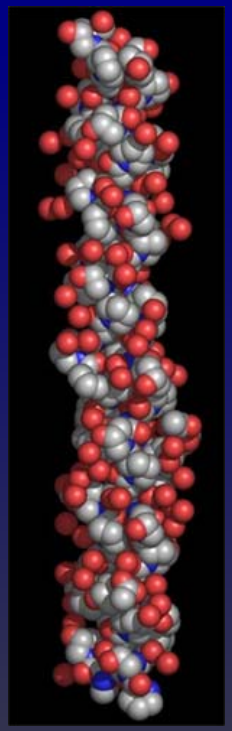
OUTLINE

- MOTIVATION
  - Collagen-based structures
  - Quantitative imaging
- BACKGROUND
  - Nonlinear optics → second-harmonic generation (SHG)
  - Basic theory of SHG
- FT-SHG
  - Approach
  - Applications
- SUMMARY
- REFERENCES

MOTIVATION AND BACKGROUND

COLLAGEN

- Accounts for 25% of total protein mass in mammals
- Molecule is 1.5-nm width, 300-nm length
- Fibrillar collagen (types I, II, III, V, XI) found in connective tissues
- Displays high degree of (supramolecular) organization
- Provides tensile strength in bones, transparency in cornea, elasticity in skin



http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=mcb.section.6542

"WISH" LIST FOR IMAGING COLLAGEN:

- High contrast/specificity
- "Good" spatial resolution
- 3D sectioning
- Minimal specimen damage
- Quantification of structural organization
- Quantification of content (molecular)
- Simple image analysis

Fourier transform-Second harmonic generation

- Minimally invasive sample prep
- Specificity to fibrillar collagen
- Inherent 3D optical sectioning

+

Fourier Analysis

- Quantify spatial variations
- Simple to use and well-established
- Phase-preserving
- Quantify content

LINEAR OPTICS

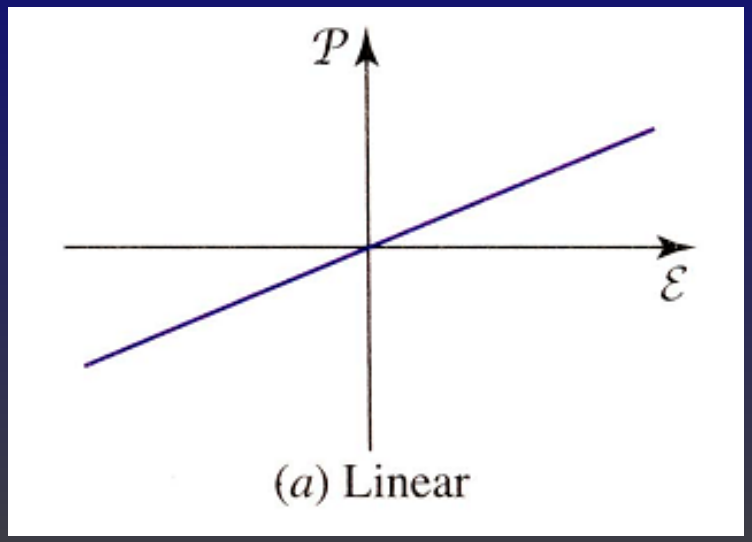
- Optical properties of materials (n and α) are independent of light intensity
- Principle of superposition applies
- Frequency of light does not change by passing through a medium
- Two independent beams of light do not interact in a medium (i.e., light cannot control light)

LINEAR OPTICS

$$\mathcal{P} = \varepsilon_o \chi \mathcal{E}$$

Polarization density

electric susceptibility



(a) Linear

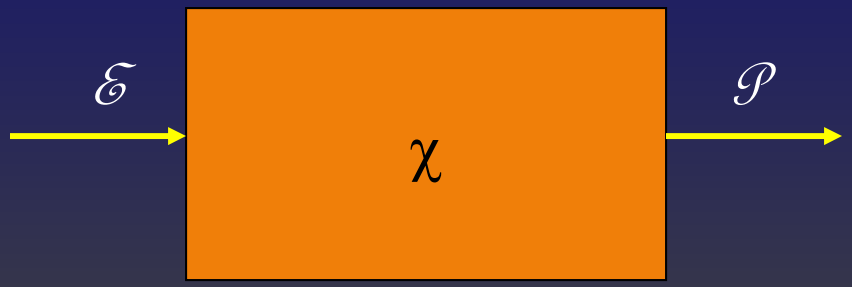


Image Source: Fundamentals of Photonics

NONLINEAR OPTICS

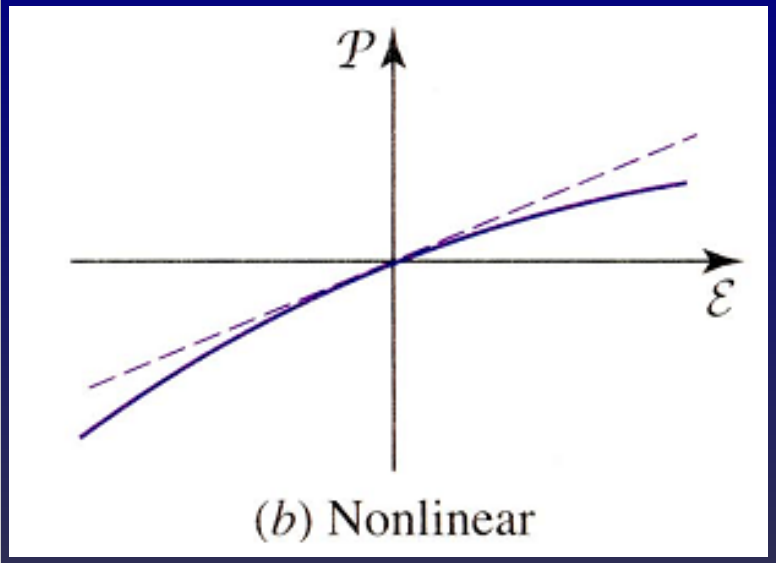
- Refractive index depends on optical intensity
- Principle of superposition does not apply in a nonlinear optical medium
- Optical frequency changes by passing through a nonlinear optical medium
- Two independent beams of light can interact within a nonlinear medium (i.e., light can control light)

WHERE IS THE NONLINEARITY?

- In the medium, not in the light
- Medium serves as a mediator for light interaction
- Dipole moment *p* could depend nonlinearly on applied electric field
- Number of atoms (or molecules) that occupy the various energy levels (during optical absorption) depends on the photon-flux density

NONLINEAR OPTICS

$$\mathcal{P}=\varepsilon_o\chi^{(1)}\mathcal{E}+\chi^{(2)}\mathcal{E}^2+\chi^{(3)}\mathcal{E}^3+\ldots$$



(*b*) Nonlinear

- Begins to deviate from linearity when $\mathcal{E}$ becomes large
- Became realizable with invention of ruby laser in 1960
- Still, externally applied e-field is small in comparison to interatomic field (~10^5-10^8 V/m), and thus nonlinearity is weak

Image Source: Fundamentals of Photonics

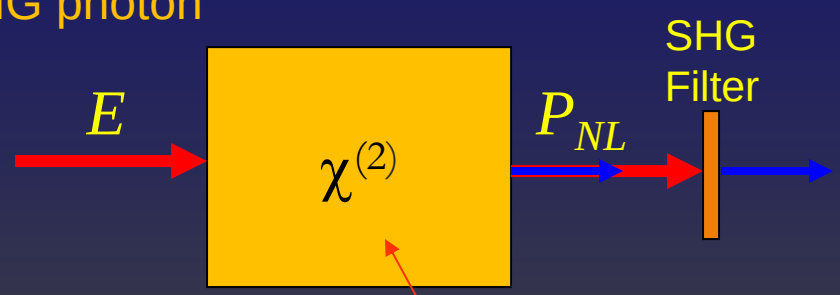
SECOND-HARMONIC GENERATION

$$P = \chi^{(1)}E + \chi^{(2)}E^2 + \chi^{(3)}E^3 + ...$$

Induced polarization

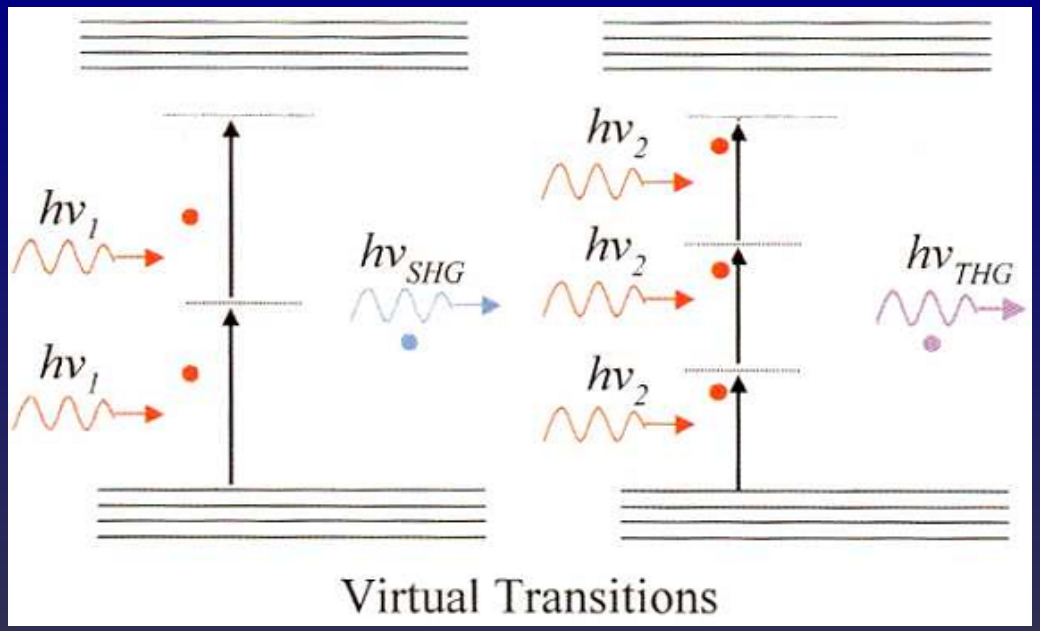
P_{NL}

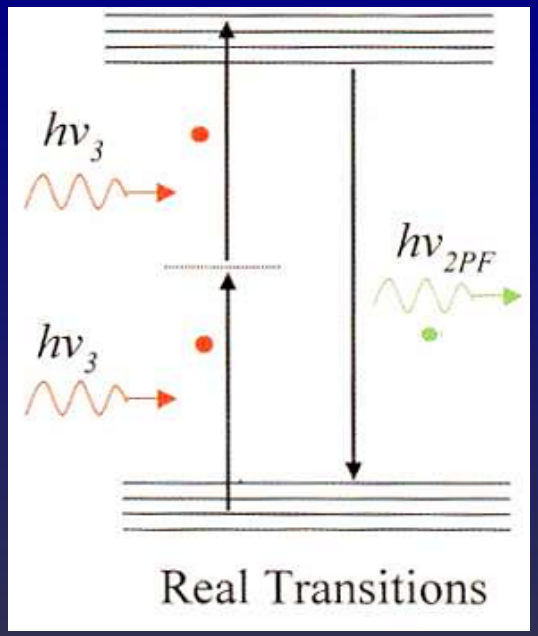
~ 1 in every billion pump photons gets converted to an SHG photon



Non-centrosymmetry

SHG: BASIC QUANTUM PICTURE



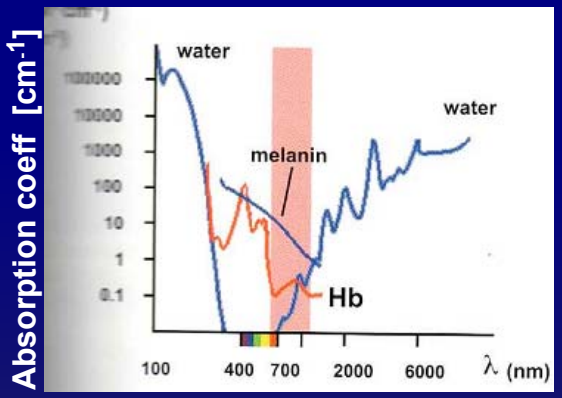


- SHG, THG → "no energy" is absorbed
- 2PF → energy is absorbed

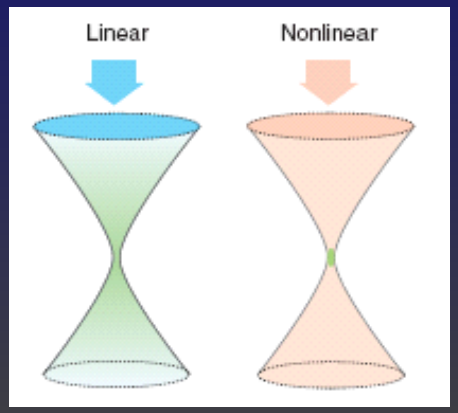
Image Source: Handbook of Biological Confocal Microscopy

SHG MICROSCOPY

- Intrinsic specificity to noncentrosymmetric structures
- Intrinsic confocality (optical sectioning)
- Deeper penetration depths than 1ph-flour (~500 μm compared to 50 μm)
- Coherent (phase preserving)

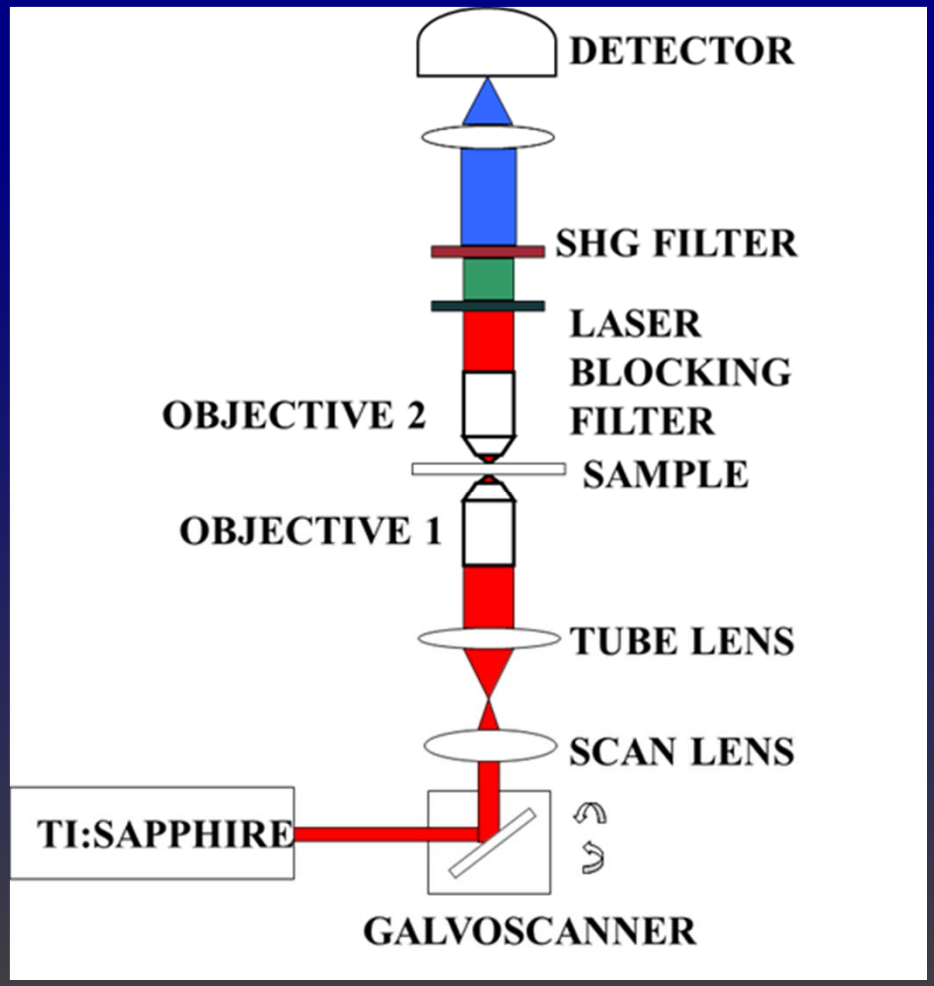


Handbook of Biological Confocal Microscopy, (2006)



Denk et al, *Nature Methods* **2**, 932 - 940 (2005)

EXPERIMENTAL SETUP



- Intrinsic specificity
- Confocality
- Phase preserving (coherent)
- Requires pulsed (typically ~ 100 fs) lasers
- Strong spatial confinement requires scanning

SAMPLE PREP

- Fixative → formalin (preserve sample from decaying)
- Usually want embedding medium (we prefer not to use any paraffin wax)
- Embed in OCT (optimal cutting temperature) compound
- Section using cryostat sectioning
- Thaw out

FT-SHG

FOURIER-TRANSFORM SHG IMAGING

- Apply Fourier transform to SHG images in 3D
- Evaluate spatial frequencies

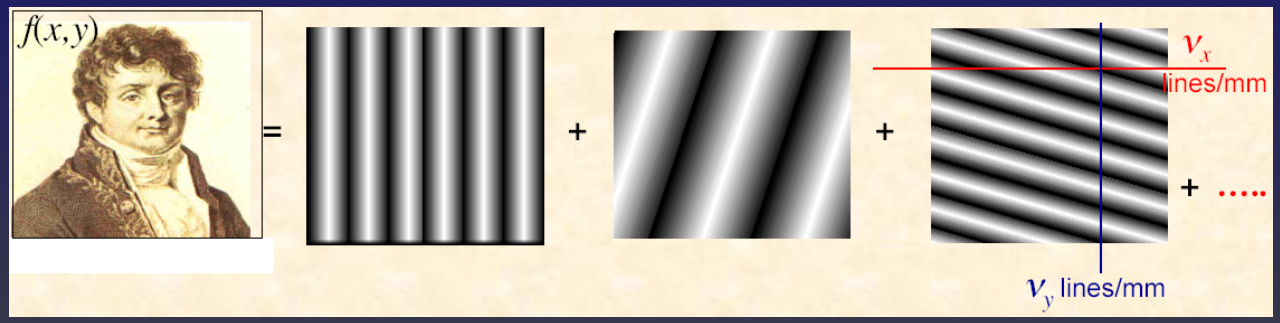
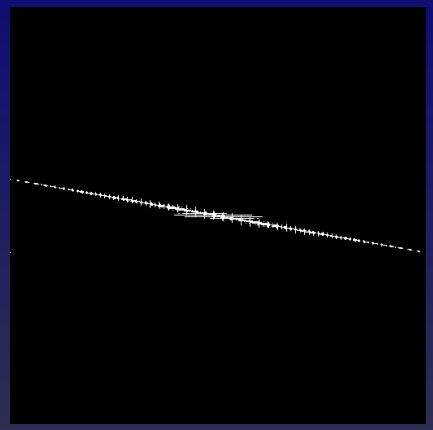


Image Source: Fundamentals of Photonics

FOURIER-TRANSFORM SHG IMAGING

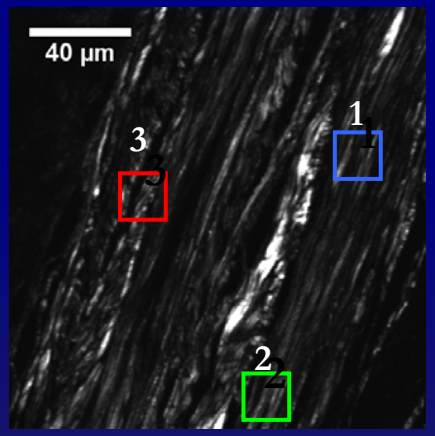
- Apply Fourier transform to SHG images in 3D
- Direction of modulation is perpendicular to preferred orientation

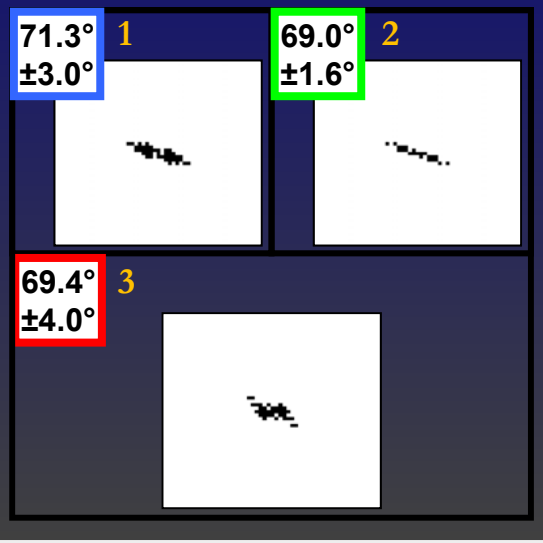
FT

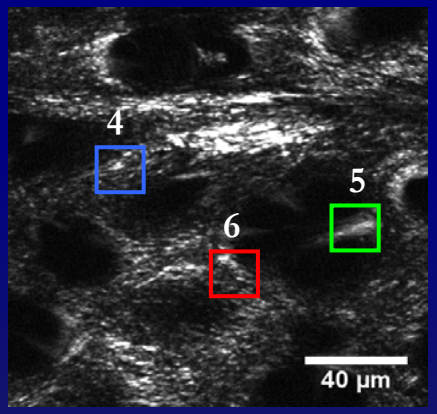


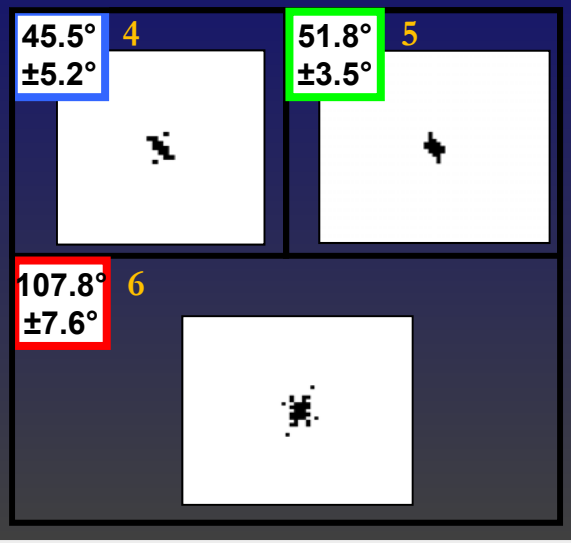
Orientation = 170°-90°=80°

PORCINE EAR AND TRACHEA





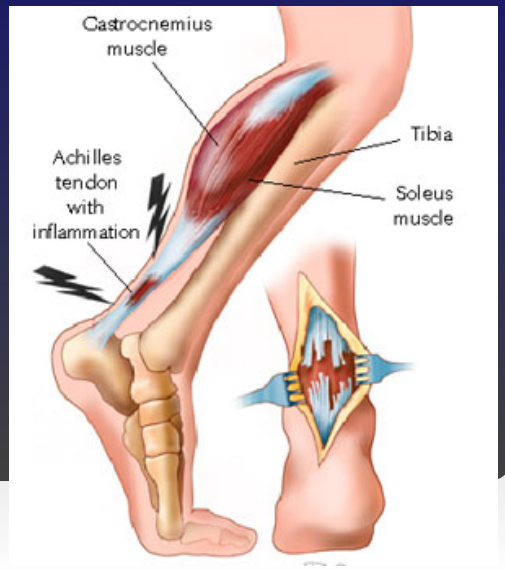




Optics Express **17**, 14534 (2009)

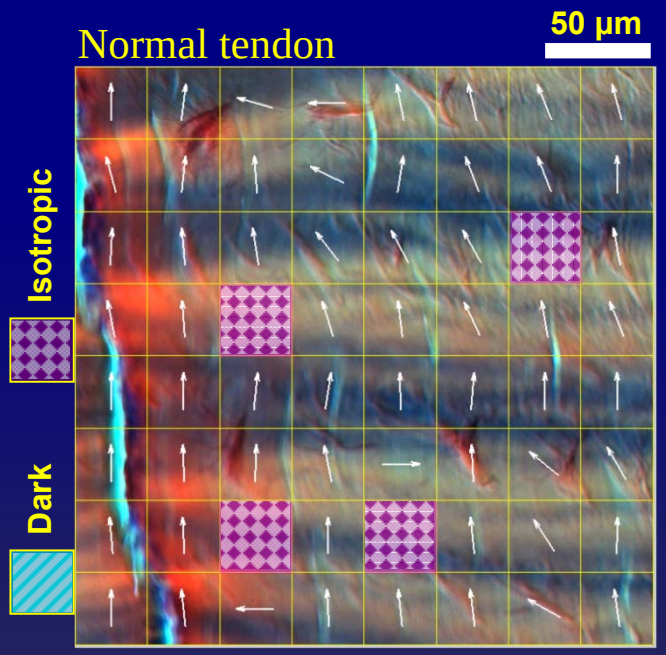
Application of FT-SHG to tendon injury (tendonitis)

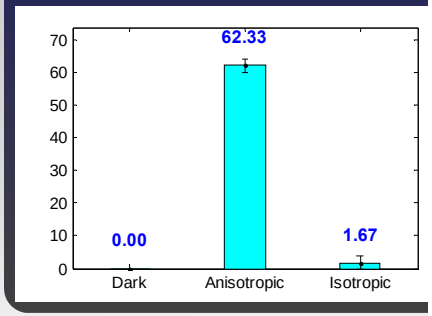
- Inflammation due to overused tendon
- Chronic pain, long rehabilitation period
- Common among athletes
- Often studied using polarization microscopy

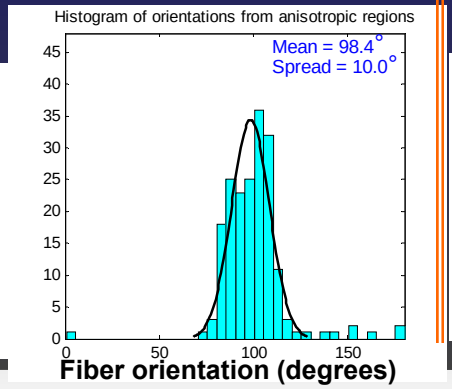


POLARIZATION MICROSCOPY OF TENDON

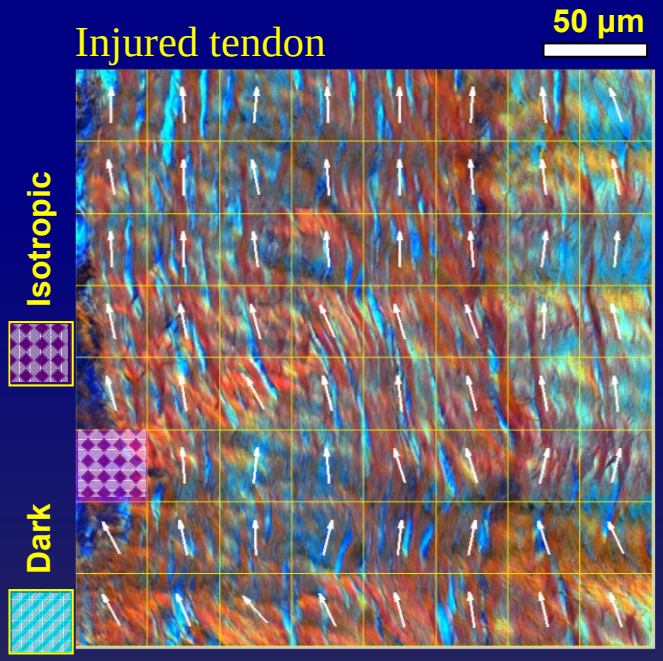
Normal tendon

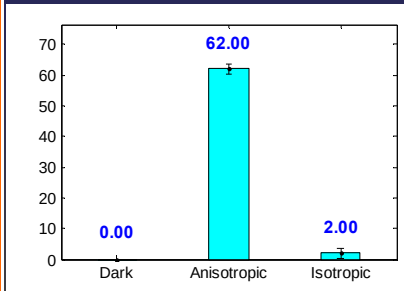


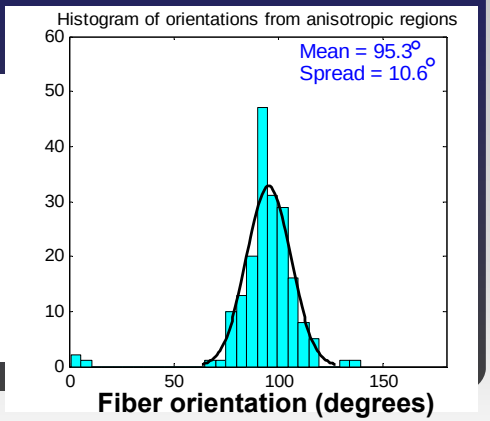




Injured tendon

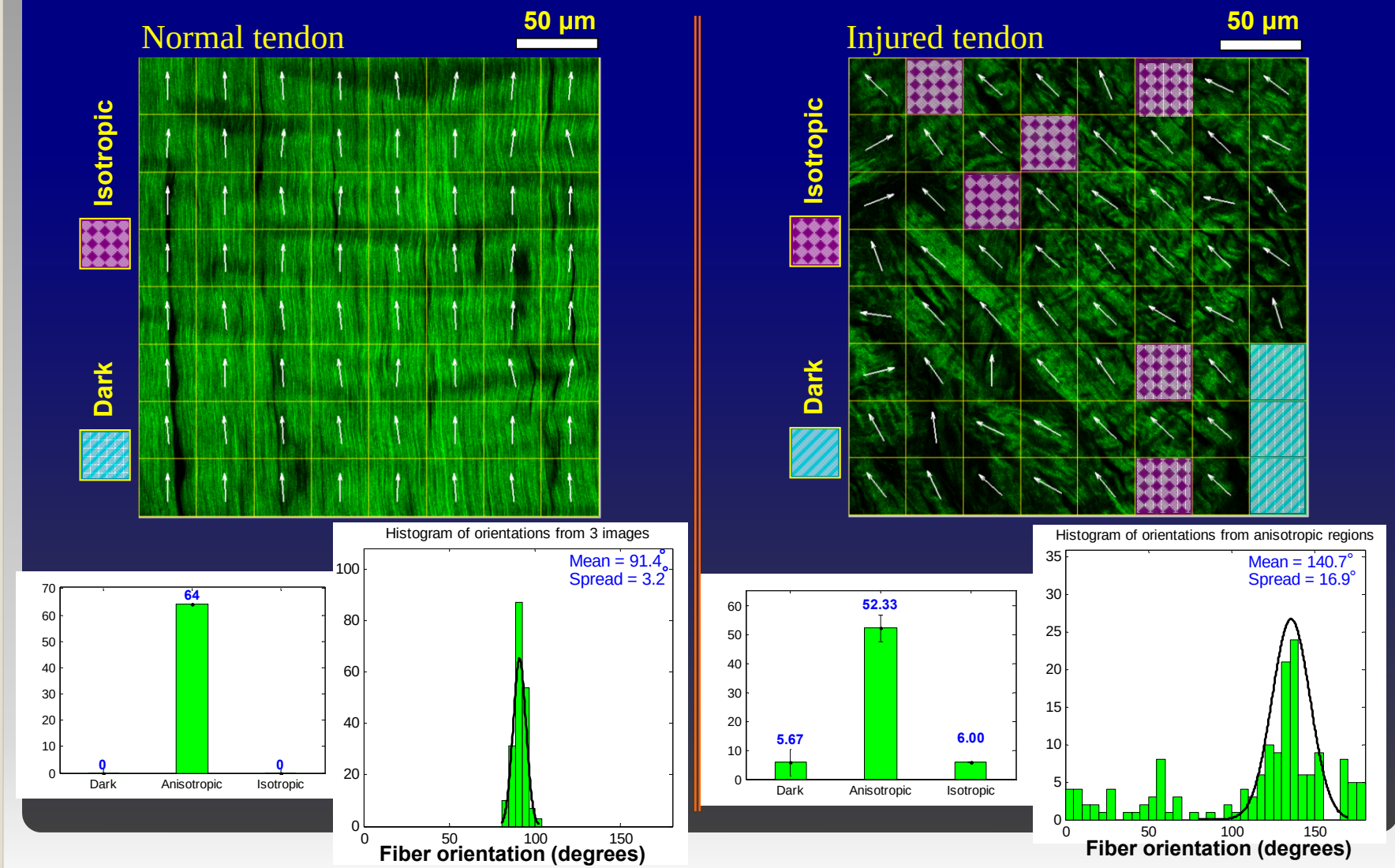






Optics Express **18,** 24983 (2010)

FT-SHG MICROSCOPY OF TENDON

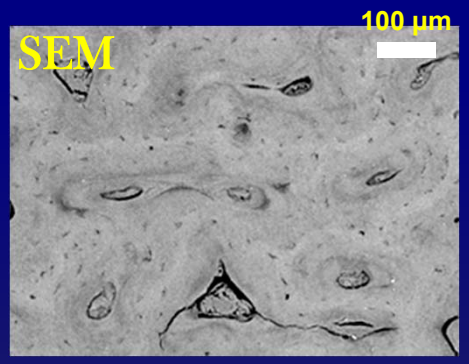


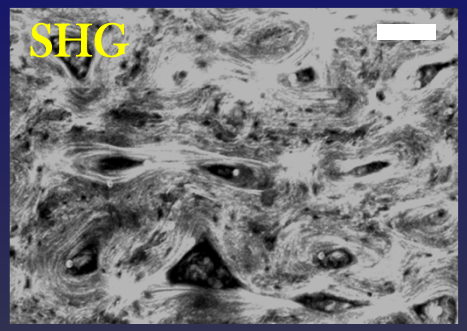
Optics Express **18,** 24983 (2010)

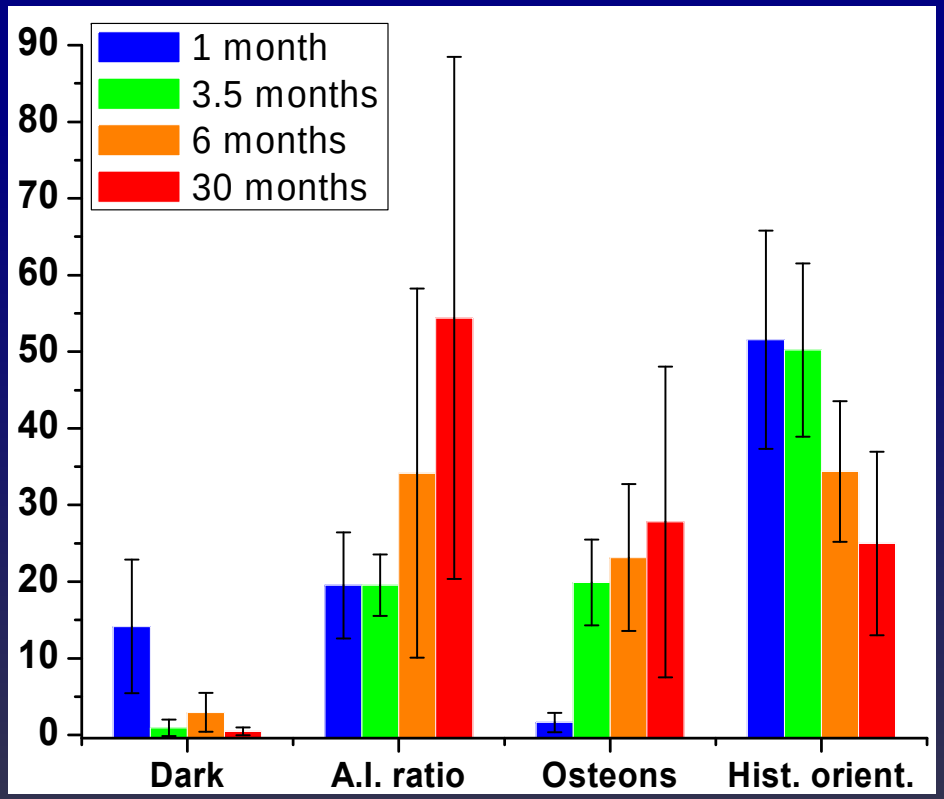
Application of FT-SHG to cortical bone structure: investigation of age-related changes

- Accounts for 80% total bone mass in adults
- Disease (osteoporosis, osteogenesis imperfecta) or injury can affect collagen structure
- SEM often used to study bone at micro to sub-cellular scales

FT-SHG OF CORTICAL BONE







- Overall reduction in porosity
- Number of osteons increases
- More organized collagen fibers comparatively

Bone **50,** 643-650 (2012)

Application of FT-SHG to Breast Biopsies

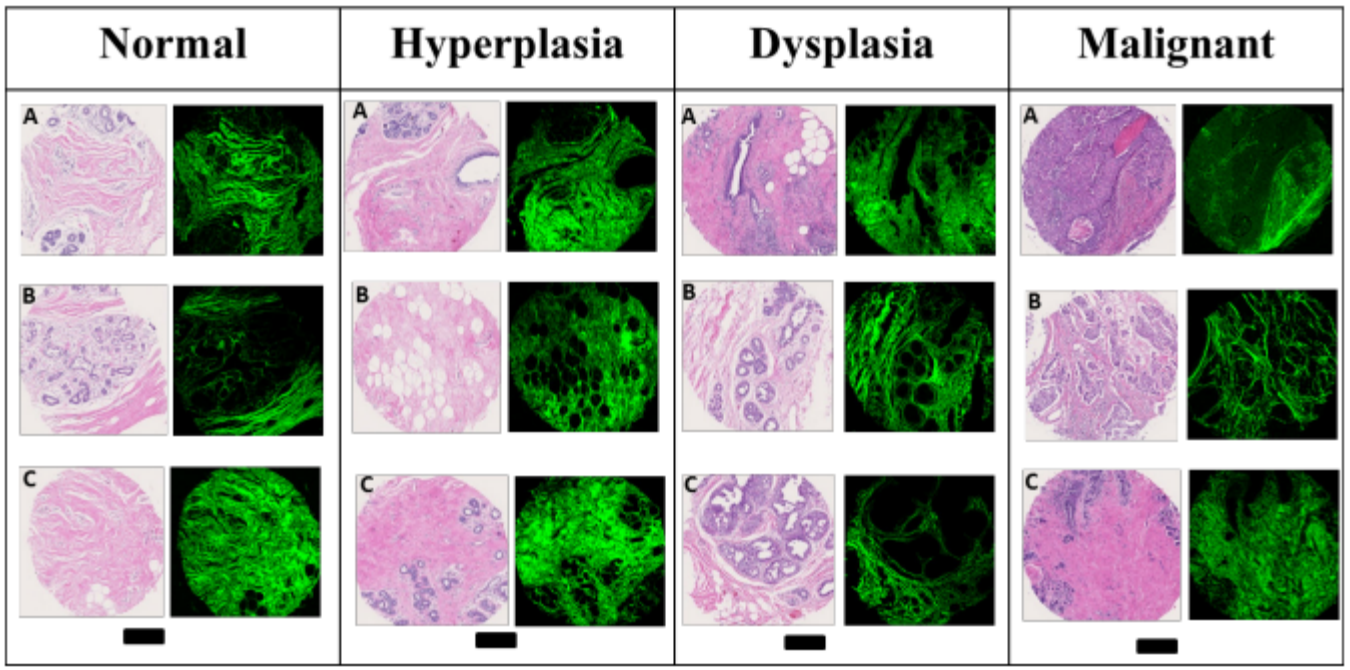
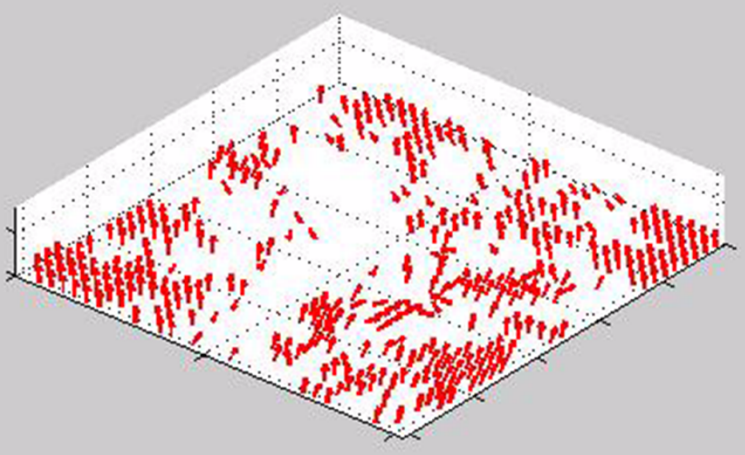
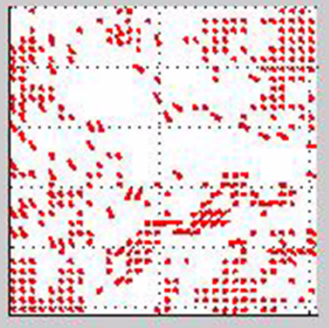


Fig. 4. H&E stained and SHG images of three cores for normal, hyperplastic, dysplastic, and malignant breast tissues. Scale bar: 250 μm.

Biomedical Optics Express 3, 2021-2035 (2012)

3D FT-SHG



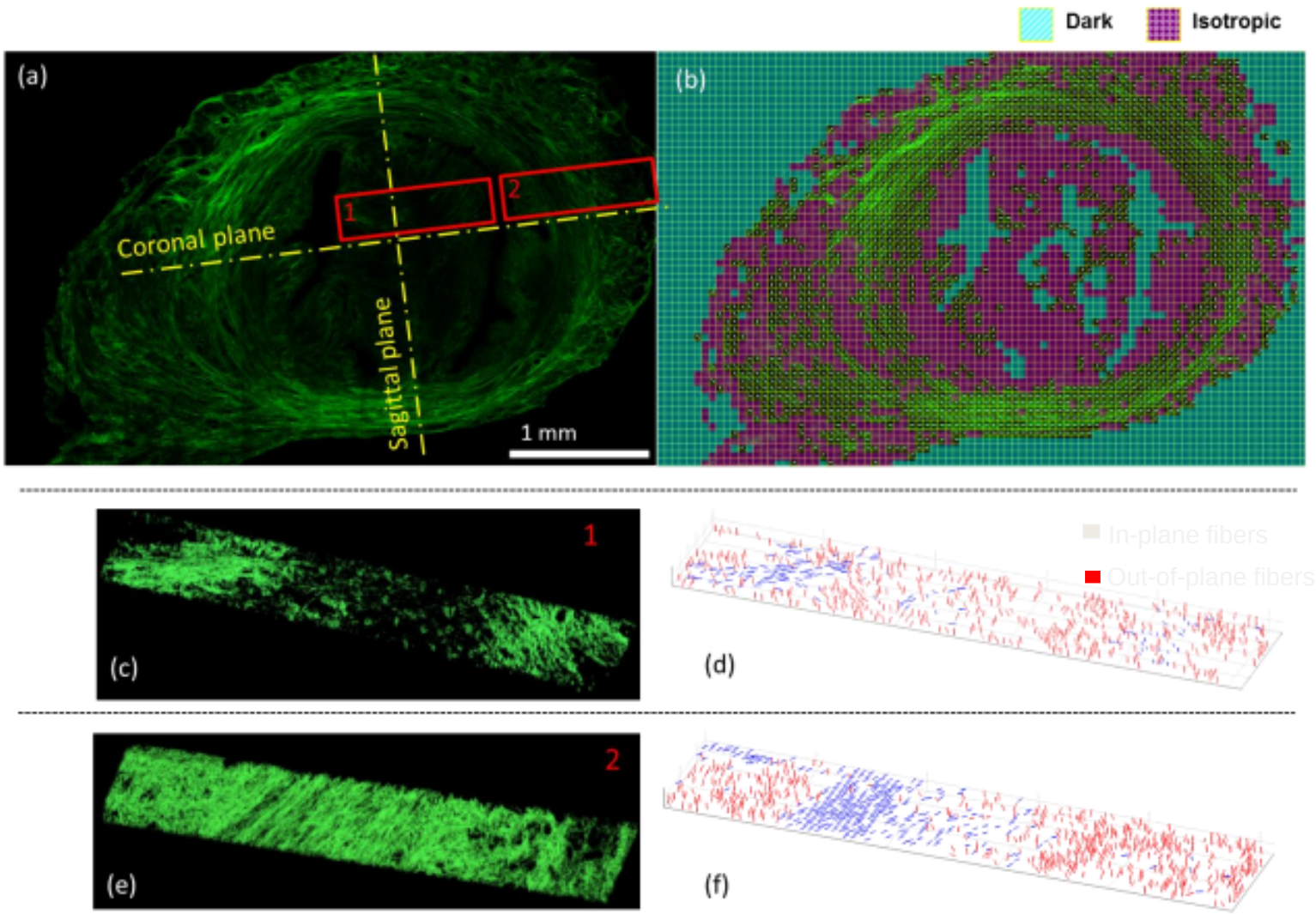


Optics Express 20, 21821-21832 (2012).

PRETERM BIRTH AND CERVIX

- 15M babies are born premature worldwide
- Long-term health effects including heart disease and diabetes
- Improved understanding of cervical remodeling (firm to soft) important
- X-ray diffraction, and electron and polarization microscopy have been used

3D FT-SHG OF RAT CERVIX



Lau et al., *Journal of Microscopy*, doi: 10.1111/jmi.12046 (2013).

SUMMARY

- FT-SHG is a simple but effective method for quantifying fibrillar collagen organization (celullar scale)
- FT-SHG was shown to have strong diagnostic potential, e.g., assessing tendon injury
- Generalized to 3 spatial dimensions with 3DFT-SHG
- 3D FT-SHG approach captures info that is hidden from 2D analysis

SOME FT-SHG REFERENCES

- Method:

  Optics Express 17, 14534-14542 (2009)
  Optics Letters, 34, 3779-3781, (2009)
  Optics Express 20, 21821-21832 (2012)

- Applications:

Tendon: *Optics Express* 18, 24983-24993 (2010)
Bone: *Bone* 50, 643-650 (2012)
Breast Biopsies: *Biomedical Optics Express* 3, 2021-2035 (2012).

(http://probe.mechse.illinois.edu)