SECOND-HARMONIC GENERATION IMAGING OF COLLAGEN-BASED SYSTEMS

presented by
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LECTURE OUTLINE

- Second-harmonic generation review
- Application of SHG imaging to qualitative studies
- Application of SHG imaging to quantitative studies
- Fourier Transform-SHG microscopy

REVIEW: NONLINEAR MICROSCOPY

- Nonlinear methods – approaches whereby output intensity is proportional to \( I^n \), where \( I \) is the input intensity and \( n \) is the number of photons involved in the interaction
  - Permits “optical histology”
  - Deeper penetration depths (~600 µm compared to 50 µm)
  - Reduced photodamage
  - Reduced photobleaching

Denk et al., Nature Methods 2, 932 - 940 (2005)
REVIEW: BASIC CONCEPT

- SHG, THG \( \rightarrow \) "no energy" is absorbed
- 2PF \( \rightarrow \) energy is absorbed

REVIEW: SHG IN BIOLOGY

- Applies to noncentrosymmetric systems, i.e., those that are highly ordered (spatially organized)
- Examples in biology include proteins: collagen, myosin, and tubulin

COLLAGEN

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

COLLAGEN (TENDON EXAMPLE)

- Tendon consists of mostly type I
- Gap (G) contains 4 collagen molecules
- 3D arrangement is not well understood


COLLAGEN AND SHG

- SHG has definitely been observed for Type I, II, III (w/I the most crystalline)
- Form 90% of types found in body
- Used to study (normal and abnormal) structure in tendon, skin, cornea, bone, etc.
- Occasionally SHG imaging combined with other modalities to increase sensitivity

QUALITATIVE APPLICATION OF SHG MICROSCOPY

- Studied collagen fiber organization in corneal stroma between human, mice, and rabbits
- Observation of bands of fibers (lamellae) in forward SHG
- Observed 2 types of lamellar organization common in all species in anterior (intervenous) and posterior (orthogonal)
- Only human showed transverse lamellae that may serve anchoring role

QUALITATIVE APPLICATION OF SHG MICROSCOPY

- SHG combined with OCT and histopathology to study wound healing in skin-equivalent tissue models (composed of type I collagen)
- Tissue exposed to thermal (laser-induced) injury
- OCT used to monitor reduction in (linear) light scattering; SHG used to monitor reduction in collagen organization

A. Yeh et al., J. Biomedical Optics 9, 248 (2004)

QUALITATIVE APPLICATION OF SHG MICROSCOPY

- SHG combined with 2PF to distinguish between various scaffold materials used in tissue engineering
- Monitored difference in emission spectrum and SHG intensity
- Alternative to using histology (and staining) for examination

Y. Sun et al., Microscopy Research and Technique 71, 140 (2008)

QUALITATIVE APPLICATION OF SHG MICROSCOPY: CLINICAL USE

- Potential for clinical use
- Development of high-NA GRIN optics permits high-resolution imaging
- Combined with 2PF microscopy

JenLab GmbH

Konig et al., Journal of Biophotonics 1, 64 (2008)
WHAT ABOUT QUANTITATIVE ANALYSIS?

- Why do we care?
- Attempt to model/quantify changes in collagen fibril organization due to physical injury or disease (lupus, Marfan syndrome, assess collagen content in tumors)
- Need to develop metrics to use as markers (correlate with morphology/physiological function)

APPLICATION OF SHG MICROSCOPY: QUANTITATIVE

- Used the ratio of forward-to-backward SHG (F/B) to study tendon collagen
- Deduced that SHG emanates from fibril shell rather than from its bulk
- F/B ratio sensitive to ionic strength of solution; results in change in shell thickness

Morphological changes are not observable from images
- Obvious changes in contrast is indicative of sensitivity to ionic concentration
- Fibril shell thickness is believed to “thin” with increasing ionic concentration
APPLICATION OF SHG MICROSCOPY: QUANTITATIVE

- Developed metrics for disorder in collagenous tissue using polarization-modulated SHG
- Disorder index
- Looked at mouse model of intervertebral disk injury
- Orientation of fibers is estimated by modulating input polarization

K. Reiser et al., Journal of Biomedical Optics 12, 064019 (2007)

APPLICATION OF SHG MICROSCOPY: QUANTITATIVE

Control disk

Disk after compressive loading

K. Reiser et al., Journal of Biomedical Optics 12, 064019 (2007)

POLARIZATION

- Often polarization microscopy is used on the same samples as SHG microscopy
- Contrasts are not the same
- Polarization microscopy is based on linear birefringence
- SHG microscopy depends on nonlinear dependence on input power
Polarization in SHG Imaging

- Used to determine matrix elements
- Provides info on degree of organization of the molecular dipoles via anisotropy parameter
- Extract orientation information

SHG and Anisotropic Materials

\[ P = \chi^{(1)}E + \chi^{(2)}E^2 + \chi^{(3)}E^3 + \ldots \]

Induced polarization

\[
\begin{bmatrix}
P_{(2\omega)} \\
P_{(2\omega)}^2 \\
P_{(2\omega)}^3
\end{bmatrix} =
\begin{bmatrix}
E_x \omega \\
E_y \omega \\
E_z \omega
\end{bmatrix}
\begin{bmatrix}
E_x(\omega) \\
E_y(\omega) \\
E_z(\omega)
\end{bmatrix}
\begin{bmatrix}
2E_x(\omega)E_x(\omega) \\
2E_y(\omega)E_y(\omega) \\
2E_z(\omega)E_z(\omega)
\end{bmatrix}
\]

Noncentrosymmetric Crystal Classes
APPLICATION OF SHG MICROSCOPY: QUANTITATIVE

- Polarization degree of freedom was used
- Ratio of $\chi^{(2)}$ elements used as a metric
- Different tissues had different collagen types (I and III)

\[
\begin{bmatrix}
0 & 0 & 0 & 0 & d_{11} & 0 \\
0 & 0 & 0 & d_{13} & 0 & 0 \\
d_{13} & d_{33} & d_{33} & 0 & 0 & 0 \\
\end{bmatrix}
\]

\[
I_{\text{obs}} \sim |\text{det}(G)| \frac{2\alpha^2}{\pi^2 c^4} \left( \frac{\chi_{11}}{\varepsilon_{22}} \cos^2 \alpha \right) \]

Determined "effective" orientation of collagen harmonophores

- Again, ratio of $\chi^{(2)}$ elements used as a metric
- This time, $\chi^{(2)}$ determined as a function of space
- Looked at human dermis and tendon-muscle junction of chicken wing

APPLICATION OF SHG MICROSCOPY: QUANTITATIVE

- Histogram distribution for $\chi^{(2)}$ for human dermis suggests detection of type III collagen in addition to type I


APPLICATION OF SHG MICROSCOPY: QUANTITATIVE (FOURIER ANALYSIS)

- SHG is excellent for imaging fibrous structures
- Fourier transforms is a useful tool for analyzing fibers/fiber arrays
- Some information about spatial organization can be obtained without the use of complex algorithms or experimental setup

FT-SHG: SPATIAL FREQUENCY

- $F_{\text{high}}$ and peaks in M.S. -> info about min. observable feature size and collagen fiber packing (spacing)
- Evaluated for direction of preferred orientation
FT-SHG: ORIENTATION

- FT picks up preferred direction of variation in image
- Creation of binary image of dominant spatial frequencies
  → apply best-fit line

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GALVOSCANNER
SCAN LENS
TUBE LENS
TI:SAPPHIRE
SAMPLE
Laser
BLOCKING FILTER
SHG FILTER
OBJECTIVE 1
DETECTOR
OBJECTIVE 2
EXPERIMENTAL SETUP

R. Ambekar et al., Optics Express 17, 14534 (2009).
FT-SHG: ORIENTATION

Porcine Ear Cartilage

(a) 71.3° ±4.0°
(b) 69.0° ±1.6°
(c) 69.4° ±4.0°
(d) 69.4° ±4.0°

R. Ambekar et al., Optics Express 17, 14534 (2009).

FT-SHG: ORIENTATION

Porcine Trachea Cartilage

(a) 45.5° ±5.2°
(b) 51.8° ±3.5°
(c) 45.5° ±5.2°
(d) 45.5° ±5.2°

R. Ambekar et al., Optics Express 17, 14534 (2009).

FT-SHG: SPATIAL FREQUENCY

More consistency in structure

~3 µm

R. Ambekar et al., Optics Express 17, 14534 (2009).
SUMMARY

- SHG is a nonlinear optical technique (coherent scattering)
- Useful in microscopy for non-invasive 3D imaging
- Recent work has focused on combining with other nonlinear imaging modes
- Development of quantitative metrics could help with assessment of tissue morphology
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http://probe.mechse.illinois.edu