SHG imaging:
From molecules to tissues

朱士維
台大物理系
Outline

- Introduction
- Principles of optical harmonics
- Experimental setup
- Applications of harmonics imaging
  - Material science
    - GaN material properties mapping
    - 3D Electric field visualization
  - Biological science
    - Bio-photonic crystal probing
    - Tissue imaging
- Summary
Optical microscopy

- **Important issues**
  - Contrast
  - Resolution
  - Penetration depth
  - Noninvasiveness

Bright field microscopy

- Human heart
- Zea mays
Advanced microscopy

- Dark field microscopy
  - ✔ Contrast enhanced
- DIC or PC microscopy
  - ✔ Contrast enhanced
- Fluorescence microscopy
  - ✔ Contrast enhanced
  - ✗ No deep tissue observation
    - ➢ Due to blurring
  - ✗ Staining required
Confocal microscopy

- Contrast enhanced
- Resolution enhanced
  - Due to the rejection of out-of-focus light
  - Optical section


J Cell Biology 105, p44 (1987)
Single photon confocal microscopy

- Inefficient collection
- Out of focus fluorescence
  - Out of focus photobleach
  - Out of focus photodamage
- Low penetration depth

\[ h\nu_1 \quad h\nu_2 \]
Two photon fluorescence (2PF) imaging

- Optical sectioning (automatically confocal)
  - High axial resolution

- Minimized out-of-focus absorption
  - Minimized out of focus photobleach/photodamage

- High penetration depth

W. Denk et al., Science 248, 73 (1990)
Problems of 2PF microscopy

- Limited penetration depth in live tissues
  (~ 150 μm @ 800 nm)
- Require in-focus two-photon absorption in labeling dye or auto-fluorescent pigment
- Photo-bleaching and photodamages
  - Due to single and multi-photon absorption with NIR
  - To fluorescent and non-fluorescent absorbers
- Limited dye penetration and toxicity issue
- Limited dye availability for structure labeling

Explore alternative spectral range and intrinsic imaging modality

Harmonics optical microscopy (HOM)

1. Denk et al., Science 248, 73 (1990)
Outline

- Introduction
- Principles of optical harmonics
- Experimental setup
- Applications of harmonics imaging
  - Material science
    - GaN material properties mapping
    - 3D Electric field visualization
  - Biological science
    - Bio-photonic crystal probing
    - Tissue imaging
- Summary
Optical harmonic generations

- Virtual transition $\rightarrow$ Energy conservation
- Resonant enhancement

Second Harmonic Generation

Third Harmonic Generation

$2h\nu_1 = h\nu_2$

$3h\nu_1 = h\nu_2$
Second harmonic generation

\[ P^{NL}(2\omega) = \frac{1}{2} \varepsilon_0 \chi^{(2)}(2\omega; \omega, \omega) E(\omega)E(\omega) \]

- \[ I(2\omega) = I(\omega)^2 \]
  - Auto-sectioning capability
- Allowed only in non-centrosymmetric media\(^1\)
  - Imaging selectivity
  - Surfaces and interface\(^2\)
  - Membrane potentials\(^3,4\)
  - Uniform polarity tissue\(^5,6\)
  - **Bio-photonic crystal effect**\(^7,8\) (structural proteins\(^9\))

1. Y. R. Shen, *The Principles of Nonlinear Optics*
Third harmonic generation

\[ P_{NL}^{3\omega} = \frac{1}{4} \varepsilon_0 \chi^{(3)}(3\omega;\omega,\omega,\omega) E(\omega)E(\omega)E(\omega) \]

- \[ I(3\omega) = I(\omega)^3 \]
  - Better sectioning capability
- Interfaces with optical inhomogeneity
  - Contour imaging

Why harmonics?

Multi-photon Fluorescence

- Optical Sectioning
- Deeper penetration due to IR wavelength

- In-focus absorption/photo-bleaching

- In-focus photo-damage

- Staining or auto-fluorescence

- Strong $\lambda$ dependency

Harmonics Generation

- No energy deposition/No absorption/photo-bleaching

- No photo-damage

- Endogenous (No staining required)

- Weak $\lambda$ selectivity
Outline

▶ Introduction
▶ Principles of optical harmonics
▶ Experimental setup
▶ Applications of harmonics imaging
  ▶ Material science
    ▶ GaN material properties mapping
    ▶ 3D Electric field visualization
  ▶ Biological science
    ▶ Bio-photonic crystal probing
    ▶ Tissue imaging
▶ Summary
Excitation wavelength selection

- Lowest attenuation around 1200 ~ 1300-nm
  - Deepest penetration in biological specimens
  - Both SHG and THG fall in visible regime
  - Reduced multiphoton fluorescence (v.s. 800-nm)
    - Reduced photodamage
  - Fiber compatible
  -Insensitive to silicon detectors
Harmonics optical microscope (HOM)

- Cr: forsterite laser
- Central wavelength: 1230-nm
- Pulse width: 130-fs
- Average power: 320-mW
- Rep. rate: 110-MHz
Outline

- Introduction
- Principles of optical harmonics
- Experimental setup
- Applications of harmonics imaging
  - Material science
    - GaN material properties mapping
    - 3D Electric field visualization
  - Biological science
    - Bio-photonic crystal probing
    - Tissue imaging
- Summary
GaN introduction

- **GaN**
  - Green-UV optoelectronic devices (LD, LED).
  - High-power/high-speed electronic devices.

- **Physical properties are strongly affected by**
  - Defect states
  - Large residue piezoelectric field due to unrelaxed strain
  - Both create spectral red-shift and is hard to distinguish in a single-point spectral measurement

GaN LED at 395 nm (LEDTronics # L200)
Motivation

- Observation of electric-field enhanced SHG in GaN\(^1\)

\[
P(2\omega) = \epsilon_0 \chi^2 (2\omega; \omega, \omega) E_{\text{laser}}^2 + \epsilon_0 \chi^3 (2\omega; \omega, \omega, 0) E_{\text{laser}}^2 E_{\text{residue}}^2
\]


- With a 1230-nm Cr:forsterite fs laser
  - SHG at 615-nm
    - Piezoelectric-field enhanced
    - Off-resonance
  - THG at 410-nm
    - Bandtail state resonant
    - Defect related

Nonlinear emission from a bulk GaN

- **SHG at 615-nm**
  - Far from GaN resonance
- **THG at 410-nm**
  - Resonant with the bandtail state
Power dependency

- Confirming 2\textsuperscript{nd} and 3\textsuperscript{rd} order nonlinearity
HOM imaging

- THG $\rightarrow$ bandtail state distribution
- SHG $\rightarrow$ piezoelectric field distribution
- Bandtail state density $\uparrow$ $\rightarrow$ piezoelectric field intensity $\downarrow$

HOM v.s. PL imaging

Bandgap luminescence (365nm)
Defect-state yellow luminescence (550-600nm)
Bandtail state
Piezoelectric field

bandgap luminescence ↓ → yellow luminescence ↑
→ defect-related bandtail state density ↑
→ piezoelectric field ↓ → strain relaxation

✗ But requires two lasers for imaging

Multiphoton excitation

5-μm bulk GaN grown on sapphire

- 4-photon fluorescence observed!
  - With a single 1230 nm source
  - 4PF in semiconductor for the first time

Resolution comparison

- The better axial resolution of 4PF over THG and SHG is demonstrated

- Peak position
  - THG: air/GaN interface
  - SHG: GaN/sapphire interface
  - 4PF: bulk contribution
Potential for spin imaging

- In GaAs/AlGaAs two dimensional electron gas
- Pump-probe SHG measurement

\[ \Delta E \text{ from gradient of electron density} \]
\[ \Delta M \text{ (spin polarization) is opposite in } +x \text{ and } -x \text{ directions} \]

Han et al., APL 91, 202114 (2007)
HOM in semiconductor

- We demonstrated laser scanning SHG, THG microscopy in bulk GaN:
  - SHG to map piezoelectric field
  - THG to map bandtail state
  - Bandtail state (defect) density $\uparrow$
    - Piezoelectric field $\downarrow$
    - Bandgap PL $\downarrow$ $\rightarrow$ Yellow luminescence $\uparrow$

- Brand new method to find out the distribution of piezoelectric field and defect state in GaN bulk and MQWs.

- Potential for spin mapping
Electrical field visualization

- **Electric probe**
  - Require metal contact
  - Invasive and indirect

- **Optical probe**
  - E-O sampling\(^1\)
    - Probe head required
    - Low 3D resolution
    - Mapping, not visualization
  - Electrical Field Induced Second Harmonic Generation (EFISHG)

Characteristics of EFISHG

Electric Field Induced Second Harmonic Generation

\[ P(2\omega) = \varepsilon_0 \chi^3 (2\omega: \omega, \omega, 0)E_{\text{laser}}E_{\text{laser}}E_{\text{applied}} \]

\[ I_{\text{EFISHG}} \propto (I_{\text{laser}})^2 \]

\[ I_{\text{EFISHG}} \propto (E_{\text{applied}})^2 \propto (V_{\text{applied}})^2 \]

- Intrinsic sectioning power \( \rightarrow \) 3D visualization
- Sub-\( \mu \)m resolution
- Ability of measuring electric field vector \( E \)

Focused laser beam

Detector

Filter
Visualize E-field by EFISHG

- **Surface EFISHG**
  - Silicon MMIC\(^1\) & Si/\(\text{SiO}_2\) heterojunction\(^2\)
  - Only at interface or surface
  - No 3D imaging capability

- **GaN EFISHG\(^3\)**
  - 3D E-field imaging
  - Strong residual SHG

- **EFISHG in liquid crystal**

HOM with EFISHG in liquid crystal

Advantages:
- High EFISHG efficiency
- Background free
- 3D E-field visualization
- Measure both amplitude and direction
- Transparent
- Non-conducting
- Easily available

Integrated-Circuit-Like Sample

Gold electrodes

10μm groove filled with non-prealigned nematic liquid crystal

Microscope cover glass

Laser
SHG confirmation

Emission spectrum of liquid crystal (30V)

\[ I_{\text{EFISHG}} \propto (I_{\text{laser}})^2 \]

Slope: 2.00
EFISHG confirmation

\[ I_{\text{EFISHG}} \propto (E_{\text{applied}})^2 \propto (V_{\text{applied}})^2 \]

Background free

\[ \chi^{3}_{xxxx} (\omega, \omega, 0) >> \chi^{3}_{xxxy} (\omega, \omega, 0) \]

Direction sensitive

Depth resolution

Resolution: $xy \sim 0.5\mu m$, $z \sim 1\mu m$
E-field visualization

- Amplitude reconstruction

\[ \sqrt{E_1^2 + E_2^2} = |E| \]

- Amplitude of electric field

- Polarization

- Gap metal
**E-field visualization**

- **Direction reconstruction**

\[
\tan^{-1} \left( \frac{E}{E} \right) = \Delta \vec{E}
\]

\[
\tan^{-1} \left( \text{polarization} \right) \div \text{polarization} = \text{Direction of electric field}
\]
Electric field in neuron

- SHG imaging for neural action potential visualization

Sacconi, PNAS 103, 3124 (2006)
Electric field in neuron

- Polarization anisotropy of SHG on neurons
- The molecular orientation is deduced

Jiang, Biophys J. 107, L26 (2007)
HOM for E-field visualization

- HOM with EFISHG in LC
  - First 3D E-field visualization
  - Sub-\( \mu \)m spatial resolution
  - Background free
  - Obtain E-field vector
    - Z-component \( \rightarrow \) sample rotation
- Action potential in neuron
Outline

- Introduction
- Principles of optical harmonics
- Experimental setup
- Applications of harmonics imaging
  - Material science
    - GaN material properties mapping
    - 3D Electric field visualization
  - Biological science
    - Bio-photonic crystal probing
    - Tissue imaging
- Summary
SHG imaging in biological tissues

- Cellulose in cell wall of maize stem
- Collagen of tendon fiber
- Starch and grana in mesophyll cells
- Myosin in muscle fiber
- Hint of crystallinity

- No labeling at all!
Nonlinear photonic crystal

\( \chi^{(2)} \) existed even with pump frequency not close to photonic bandgap.

Broderick, PRL 84, 4345 (2000)
Dumeige, APL 78, 3021 (2001)
Nonlinear bio-photonic crystal

- First observed in bR (≈ 5-nm period)
- Strong SHG is observed
  - No SHG after bR was hydrolyzed

Lots of orderly-arranged nano-structure in biology
- Stacked membranes: starch granule, grana, mineral deposition
- Arrayed microtubules: cellulose microfibrils, myofibrils in a muscle fiber, and collagen bundles, etc.

Can be studied by SHG

Nonlinear biophotonic crystal

Mitosis spindle of a zebrafish blastoderm  THG + SHG

- **SHG**
  - Crystallized microtubule array
  - Diminished after the microtubules dispersed

- **THG**
  - Cellular and nuclear plasma membranes
Can we find the arrangement symmetry of underlying molecules by SHG?
  - Active molecule identification
  - Molecular structural/packing information elucidated
SHG of starch

Bright field  Polarized microscope  SHG
Molecular origin of starch SHG

- **Amylopectin** or **Amylose**?

3. In preparation to *Biophys J*

Japonica rice
Amylopectin: 86%
Amylose: 14%

Japonica waxy rice
Amylopectin: 99%
Amylose: 1%

SHG from Japonica waxy rice is 15% stronger

**SHG from amylopectin!!**
SHG of starch

- Full $\chi^{(2)}$ tensor and molecular orientation are deduced

$$
SHG \propto \left( \chi^{(2)}_{16} \sin 2\theta \right)^2 + \left( \chi^{(2)}_{21} \sin^2 \theta + \chi^{(2)}_{22} \cos^2 \theta \right)^2
$$

$$
\chi^{(2)} = \chi^{(2)}_{16} \begin{bmatrix} 0 & 0 & 0 & 0 & 0 & 0 & 1 \\ 0.23 \pm 0.09 & 0.95 \pm 0.04 & 0.23 \pm 0.09 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 1 & 0 & 0 & 0 \end{bmatrix}
$$
SHG in muscle

- From myosin filaments, not actin

2D Bio-photonic crystal in animal

Muscle fibers

- Full $\chi^{(2)}$ tensor is resolved
  - Based on cylindrical symmetry assumption

\[ \chi^{(2)} = \chi^{(2)}_{31} \begin{bmatrix} 0 & 0 & 0 & 0 & 1.15 & 0 \\ 0 & 0 & 0 & 1.15 & 0 & 0 \\ 1 & 1 & 0.09 & 0 & 0 & 0 \end{bmatrix} \]

SHG from myosin

- Polarization anisotropy
  - SHG from coiled-coil filaments of myosin
  - The inclination angle of molecular coil is determined by fitting the anisotropy
    - 61.2 deg, matching X-ray diffraction results

SHG anisotropy

- Muscle

- Collagen

Selective imaging by SHG

- Biological tissues usually entangle with each other
  - e.g. muscle fiber & collagen-based endomysium
- Both exhibit strong SHG
- How to selectively observe them without staining?
Polarization based selective imaging

Over 100-fold contrast enhancement

Chu, *APL* 91, 103903 (2007)
Emission dipole based selective imaging

- Muscle fibers: FSHG dominated
- Collagen: both FSHG and BSHG

BSHG vs. FSHG

- They do not overlap well
  - BSHG does not come merely from backscattering
BSHG vs. FSHG

- Thickness determination in a collagen fibril

 Thickness of a collagen fibril

- Determined by FSHG/BSHG ratio
- Ten nanometer precision

![Diagram showing the correlation between active cluster size (nm) and collagen fibril thickness (nm)]
Virus imaging

- No labeling is required

Normal cells

Infected cells

Nuclear polyhedrosis viruses in living cells

SHG to locate the virus

THG to outline the cells

SHG polarimetry

- Body-centered-cubic arrangement of polyhedrin trimers was found from the virus
Future prospect

- SHG is sensitive to molecular structure
  - Membrane / thin-film study
  - Spin dynamics mapping
  - Electric field visualization
  - Thermal effect probing
  - Deep tissue imaging
Summary for HOM

- **Issues of optical microscopy**
  - **Contrast**
    - Greatly enhanced
    - Function/structure specificity
  - **Resolution**
    - 300-nm for THG, 400-nm for SHG in our case
  - **Penetration depth**
    - > 1.5-mm
  - **Noninvasiveness**
    - Long-term embryonic observation
    - No exogenous labeling
Summary for HOM

- Very good candidate for
  - Material characteristics mapping
  - E-field 3D visualization
  - Bio-photonic crystal probing
  - Developmental biology
  - And much more……..
Acknowledgement

- **My lab**
  - 卓宗衍
  - 游鈞彥
  - 曾鈺懿
  - 廖建盛

- **UFO/NTU**
  - 孫啟光教授
  - 陳嘉維博士
  - 劉子銘博士
  - 戴世芃博士
  - 廖建盛

- **台大漁科所**
  - 蔡懷禎教授
  - 林正勇

- **生物技術開發中心（DCB）**
  - 林白翎博士
  - 陳勇志
  - 陳振銘

- **陽明大學**
  - 林奇宏教授
  - 蕭一清博士
  - 何佳霖

- **UCSB**
  - Prof. S.P. Denbaars
  - P. Fini

- **NYSU/Buffalo**
  - 鄭炳今教授

Thank you!