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

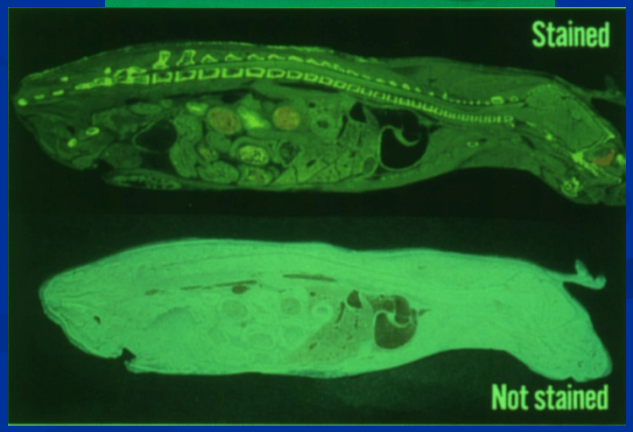
Optical microscopy

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

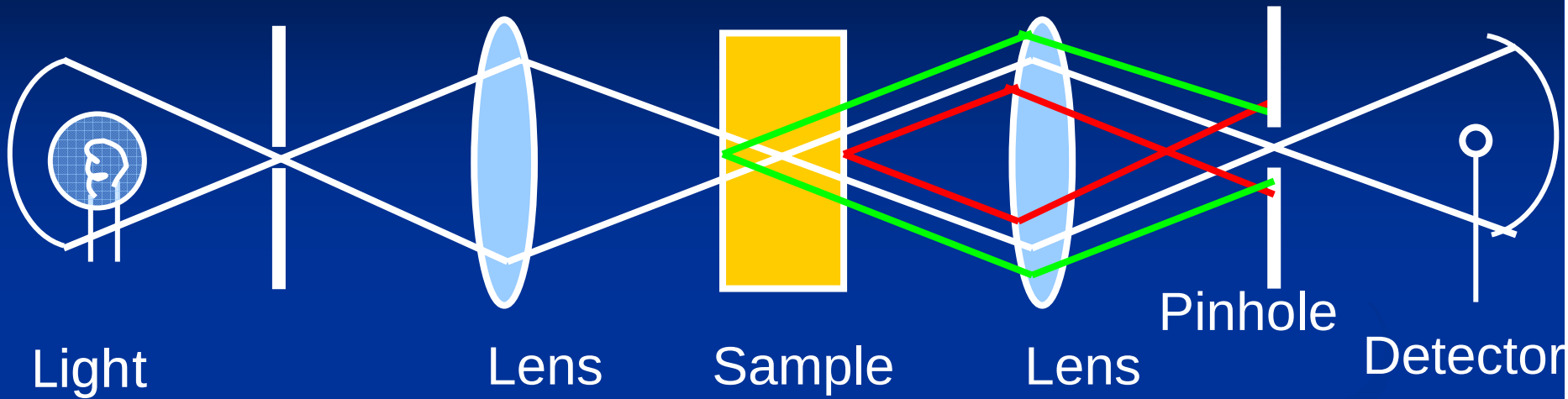
Bright field microscopy

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

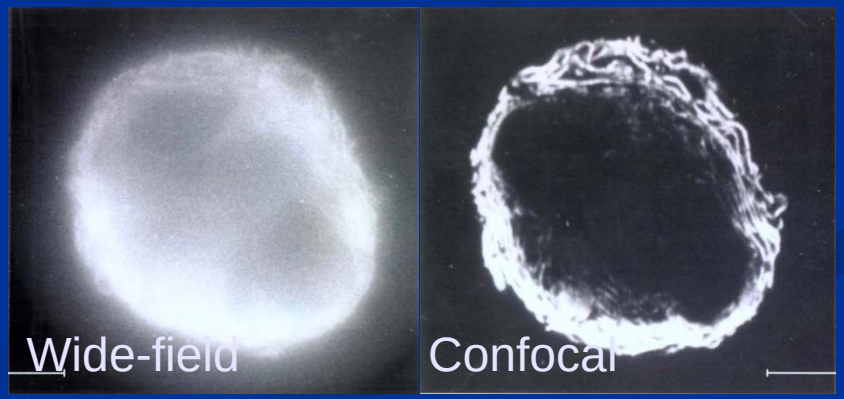


Confocal microscopy



M. Minsky, *US Patent 3013467* (1961)

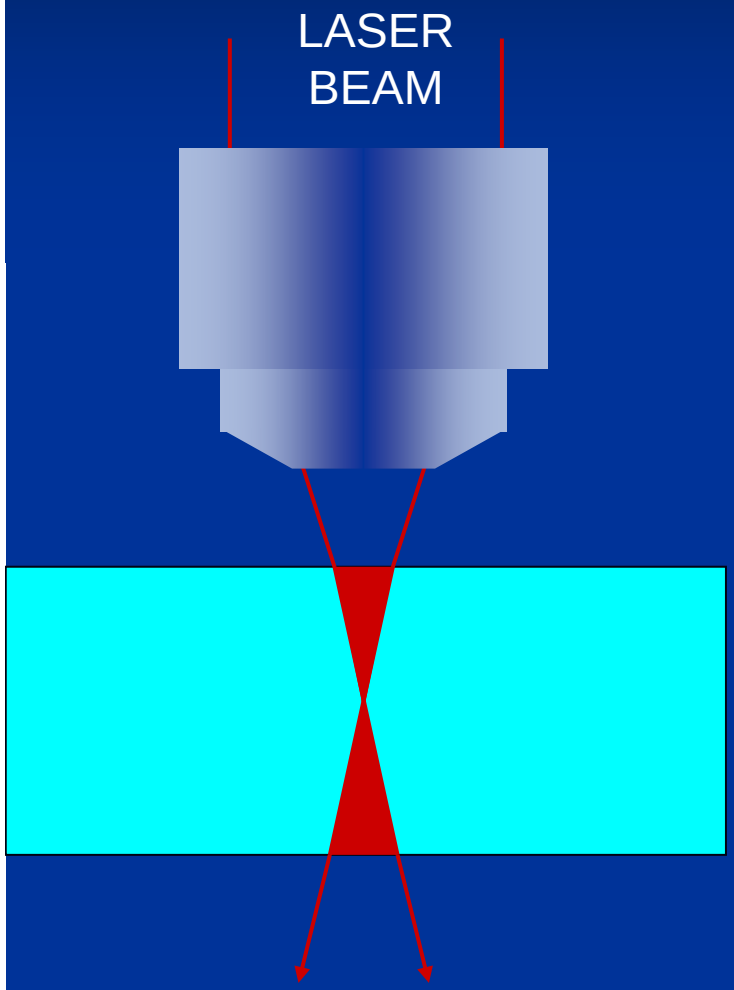
- Contrast enhanced
- Resolution enhanced
  - Due to the rejection of out-of focus light
  - Optical section



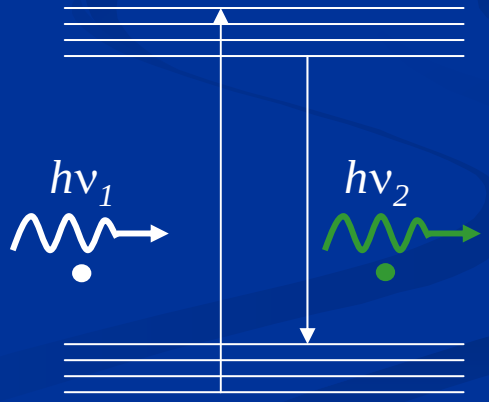
Plasmacytoma cell

J Cell Biology 105, p44 (1987)

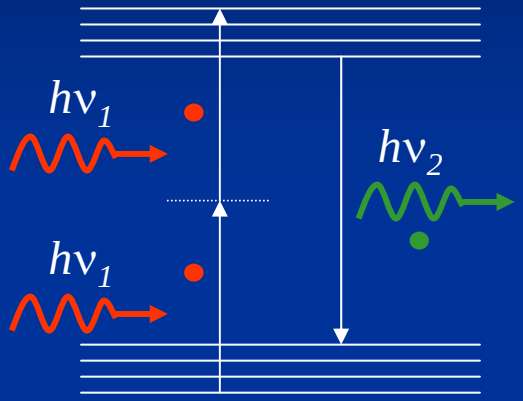
Single photon confocal microscopy

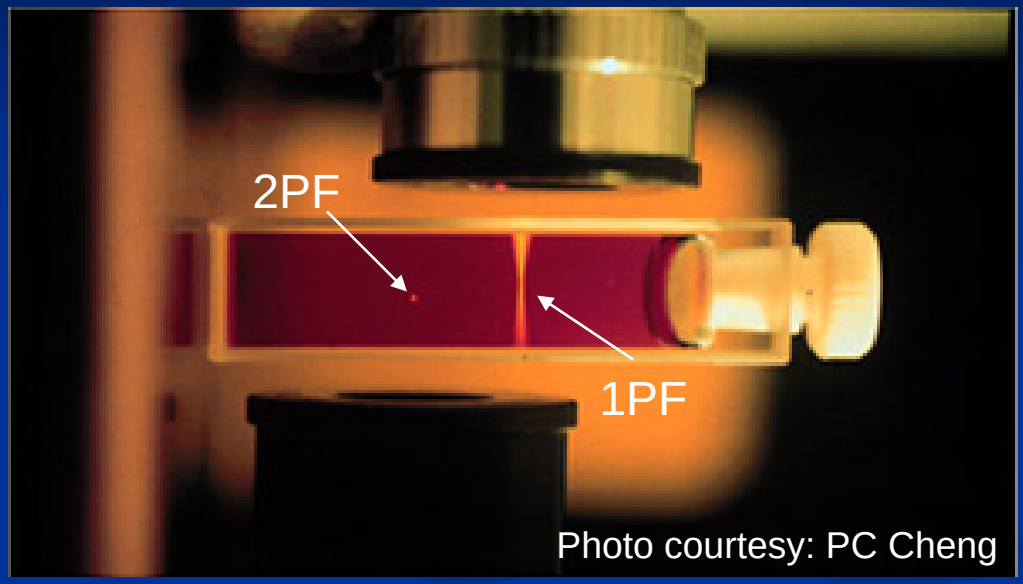


- Inefficient collection
- Out of focus fluorescence
  - Out of focus photobleach
  - Out of focus photodamage
- Low penetration depth



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[1]
- Photo-bleaching and photodamages
  - Due to single and multi-photon absorption with NIR[2]
  - 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)**

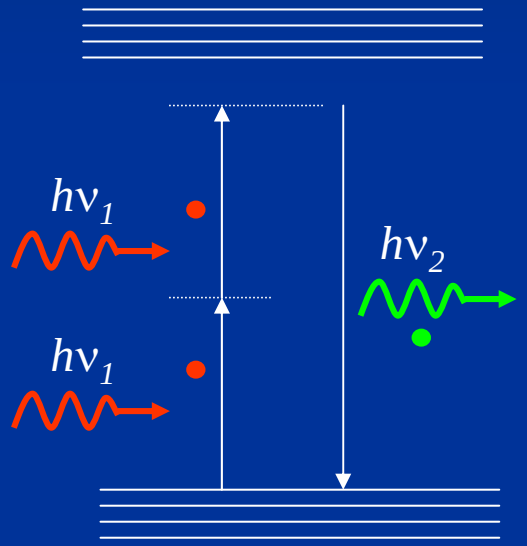
1. Denk *et al.*, *Science* 248, 73 (1990)
2. König *et al.*, *Opt. Lett.* 22,135 (1997)

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

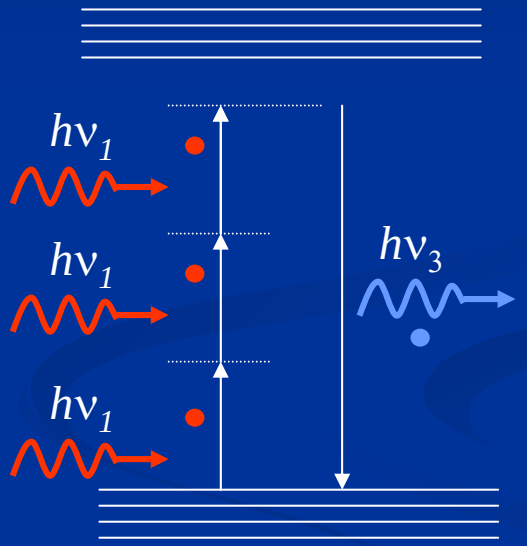
Optical harmonic generations

- Virtual transition → Energy conservation
- Resonant enhancement



Second Harmonic Generation

$2h\nu_1 = h\nu_2$



Third Harmonic Generation

$3h\nu_1 = h\nu_2$

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*
2. Y. R. Shen, *Nature* 337, 519(1989)
3. L. Moreaux *et al*., *Opt. Lett.* 25, 320 (2000).
4. G. Peleg, *et al*., *PNAS*. 96, 6700 (1999).
5. I. Freund *et al*., *Biophys. J*. 50, 693 (1986).
6. Y. Guo, *et al*., *Opt. Lett.* 22, 1323 (1997).
7. S.-W. Chu, *et al*., *Opt. Lett.* 26, 1909 (2001).
8. S.-W. Chu, *et al*., *J. Microscopy* 208, 190 (2002).
9. P. J. Campagnola *et al*., *Biophy. J.* 81, 493 (2002).

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

D. Yelin and Y. Silberberg, *Opt. Express* 5, 169 (1999)

M. Muller *et al*., *J. Microscopy* 191, 266 (1998).

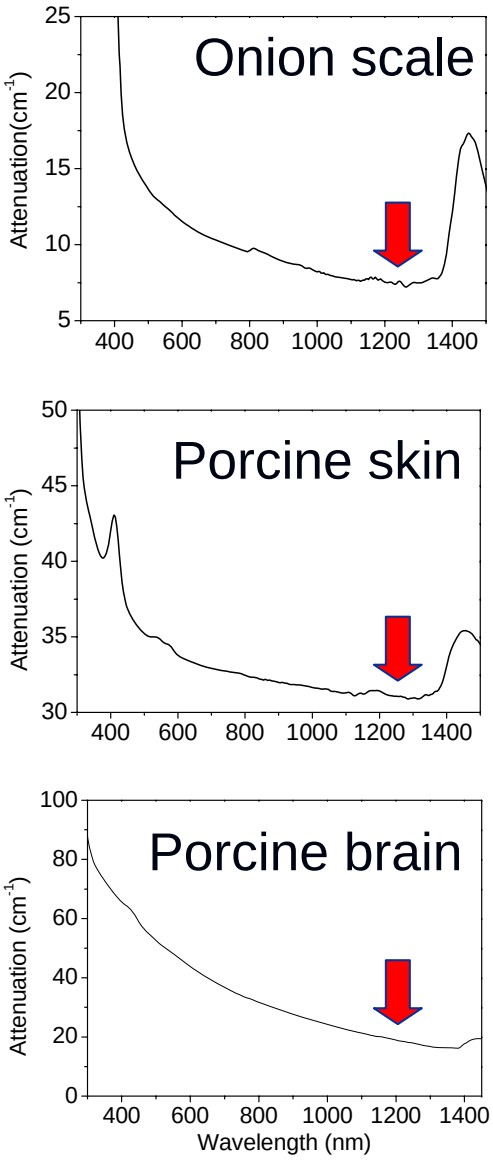
Why harmonics?

Multi-photon Fluoresecence	Harmonics Generation
	✓ Optical Sectioning
	✓ Deeper penetration due to IR wavelength
✘ In- focus absorption/photo-bleaching	✓ No energy deposition/No absorption/photo-bleaching
✘ In-focus photo-damage	✓ No photo-damage
✘ Staining or auto-fluorescence	✓ Endogenous (No staining required)
✘ Strong λ dependency	✓ Weak λ selectivity

Outline

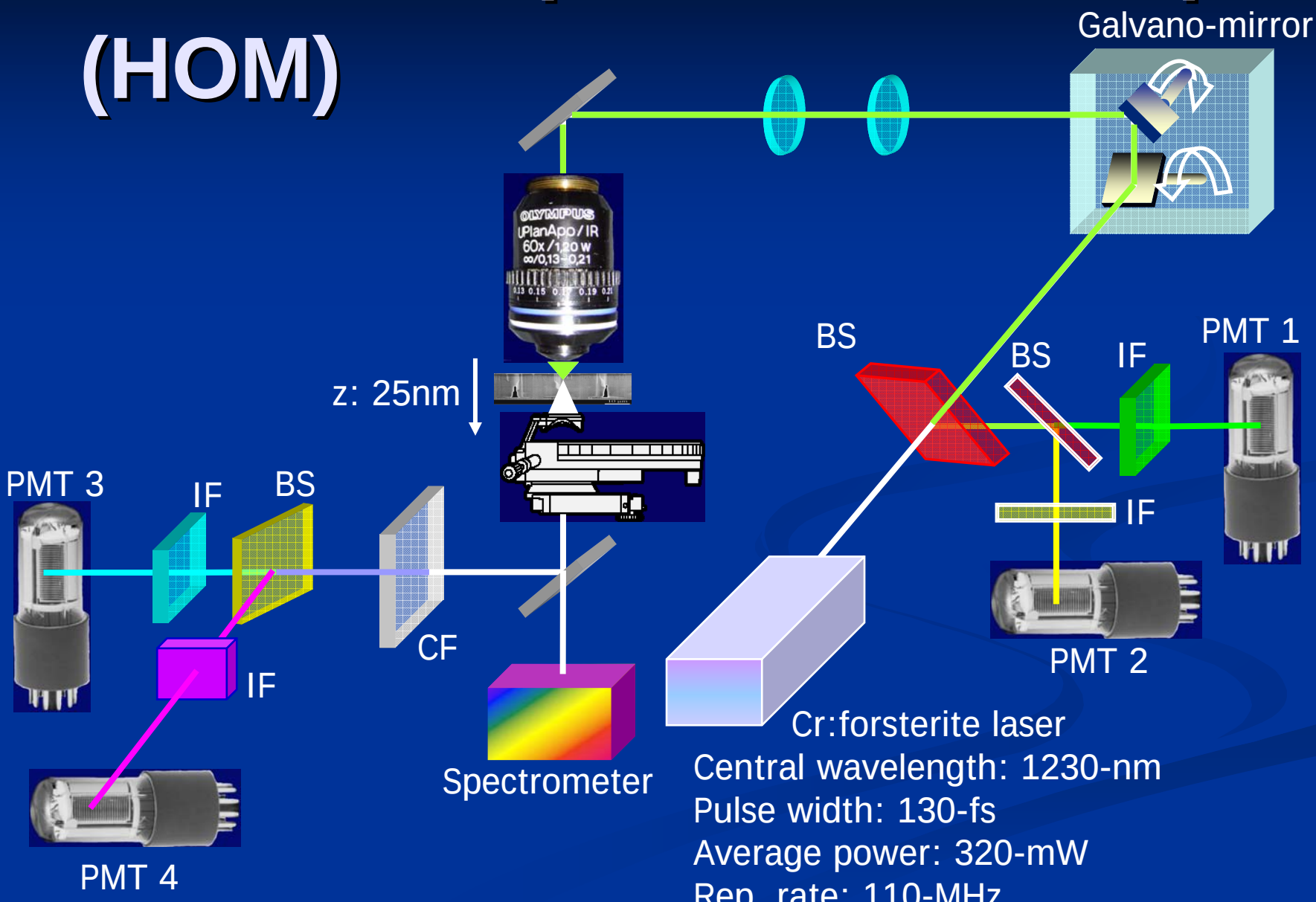
- Introduction
- Principles of optical harmonics
- Experimental setup
- Applications of harmonics imaging
  - Material science
    - GaN material properties mapping
    - 3D Electric field visualization
  - Biological science
    - Bio-photonic crystal probing
    - Tissue imaging
- Summary

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)



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)

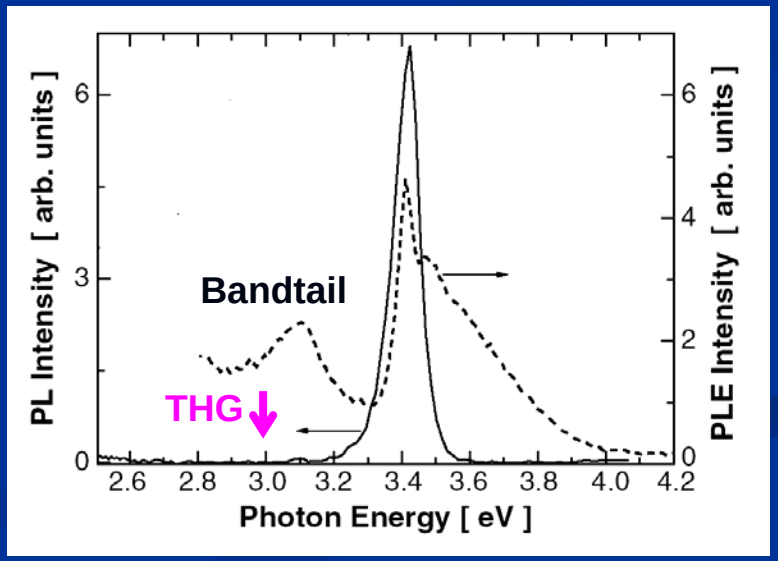
Motivation

- Observation of electric-field enhanced SHG in GaN[1]

$$P(2\omega) = \varepsilon_0 \chi^{(2)}(2\omega:\omega,\omega)E_{laser}E_{laser} + \varepsilon_0 \chi^{(3)}(2\omega:\omega,\omega,0)E_{laser}E_{laser}E_{residue}$$

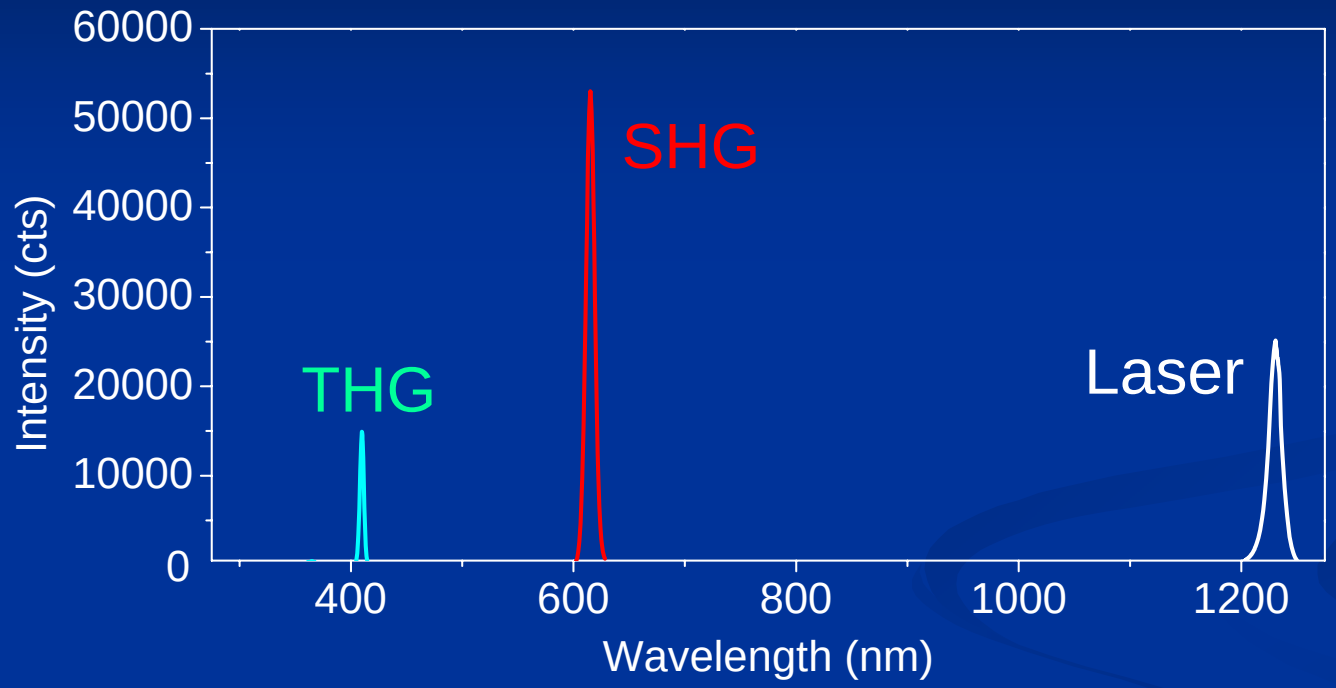
J. Miragliotta & D. K. Wickenden, Phys. Rev. B, v. 53, 1388 (1997).

- 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



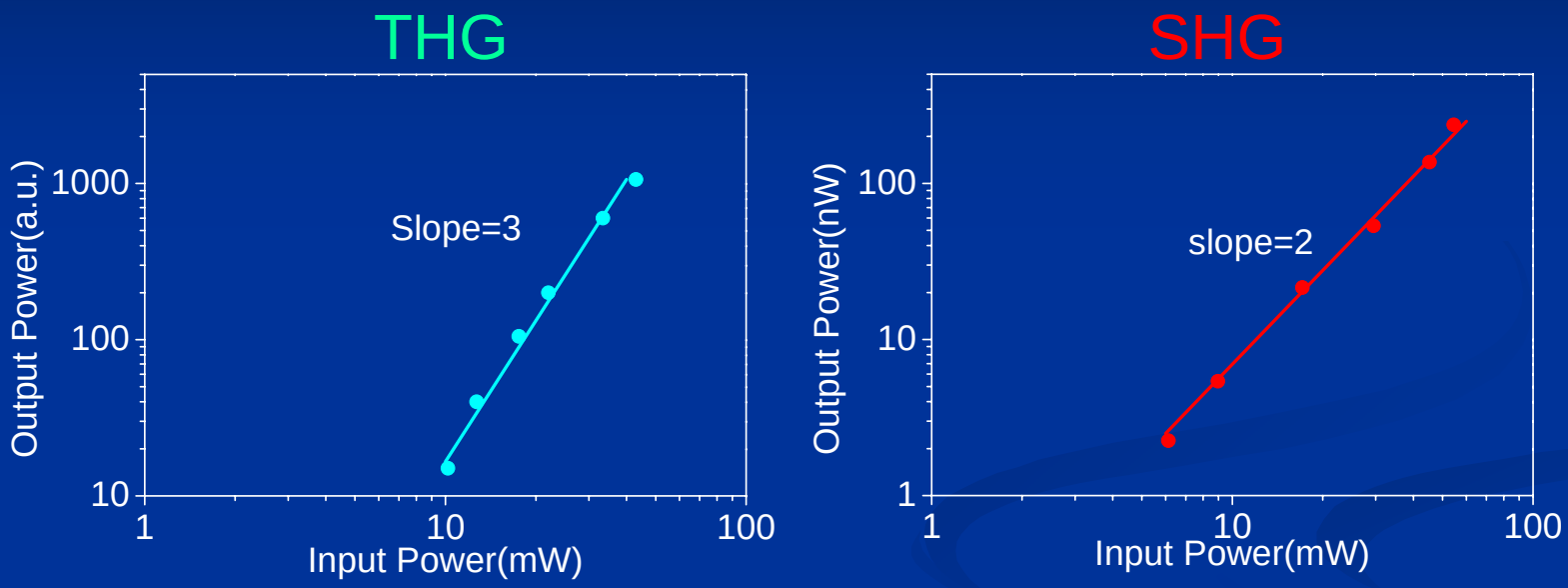
B. Guo et al., Appl. Phys. B 80, 521 (2005).

Nonlinear emission from a bulk GaN



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

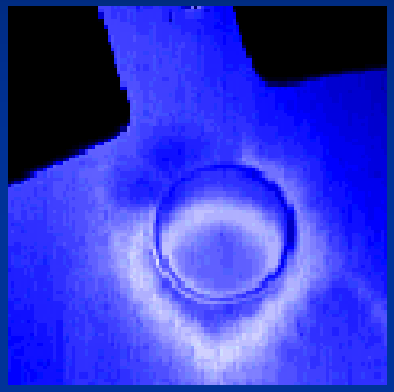
Power dependency



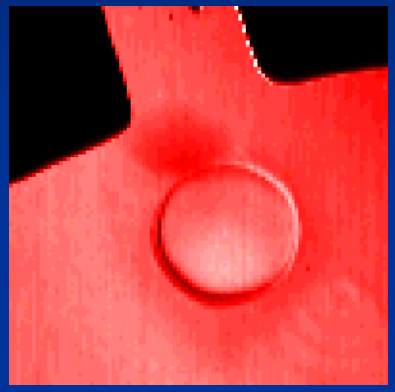
- Confirming 2nd and 3rd order nonlinearity

HOM imaging

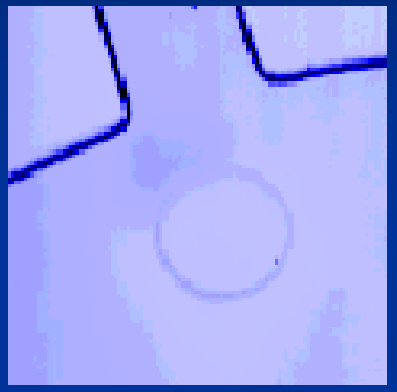
THG



SHG



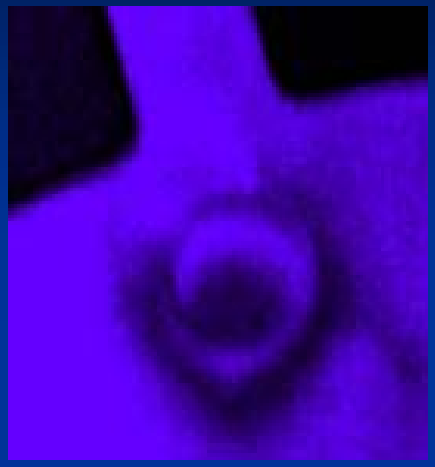
IR Transmission



- THG → bandtail state distribution
- SHG → piezoelectric field distribution
- Bandtail state density ↑ → piezoelectric field intensity ↓

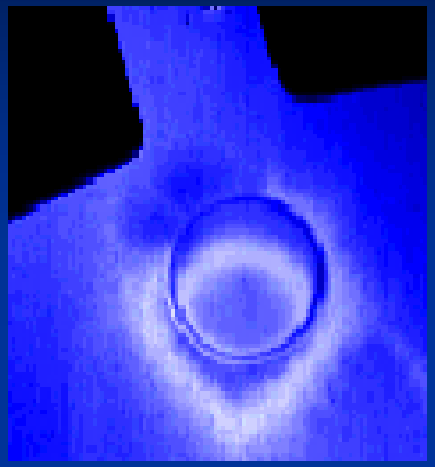
C.-K. Sun & S.-W. Chu et al., *APL* 77, 2331-2333 (2000)
C.-K. Sun & S.-W. Chu et al., *Scanning* 23, 182-192, **invited paper** (2001)

HOM v.s. PL imaging

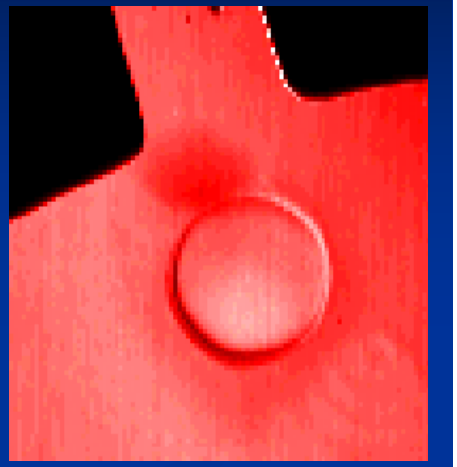


Bandgap luminescence (365nm)

Defect-state yellow luminescence (550-600nm)



Bandtail state



Piezoelectric field

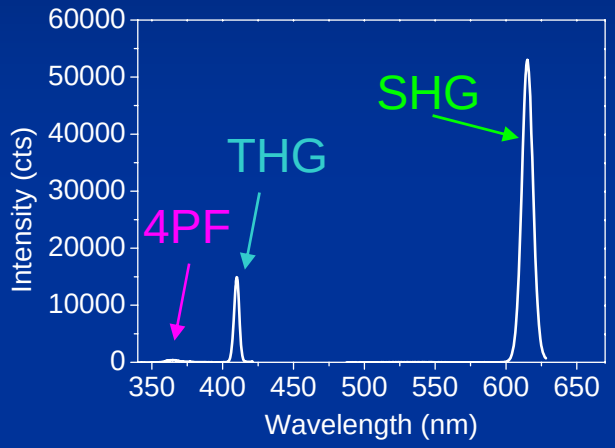
bandgap luminescence ↓ → yellow luminescence ↑
→ defect-related bandtail state density ↑
→ piezoelectric field ↓ → strain relaxation

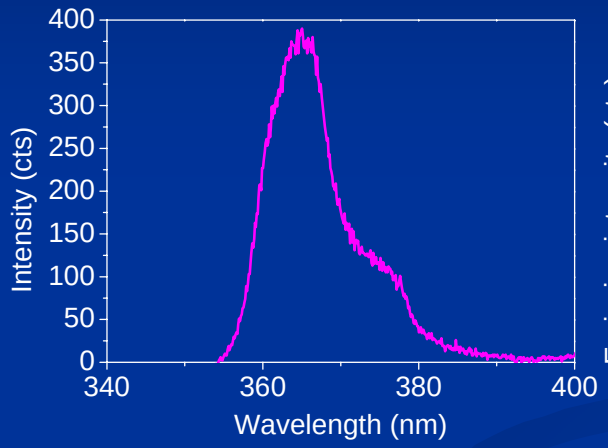
- But requires two lasers for imaging

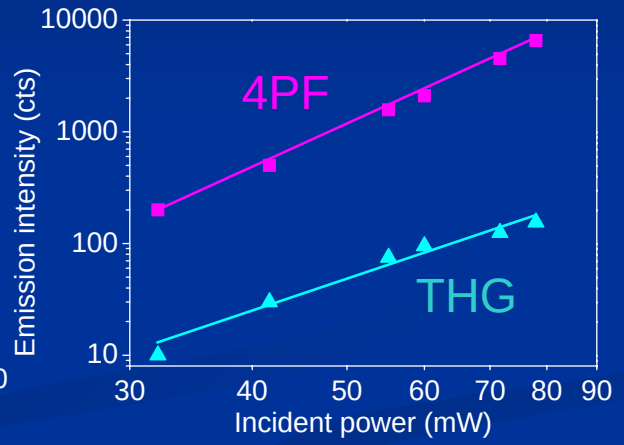
C.-K. Sun et al., APL 76, 439 (2000).

Multiphoton excitation

5-μm bulk GaN grown on sapphire



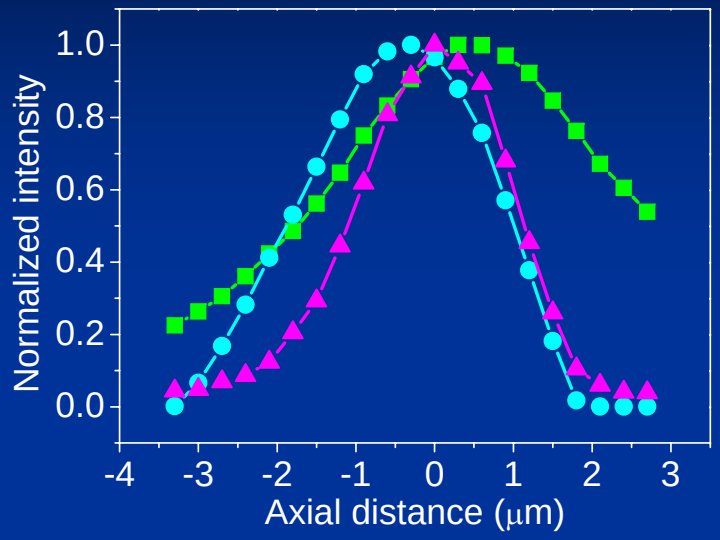




- 4-photon fluorescence observed!
  - With a single 1230 nm source
  - 4PF in semiconductor for the first time

S. W. Chu, *Opt. Lett.* **30**, 2463-2465 (2005)

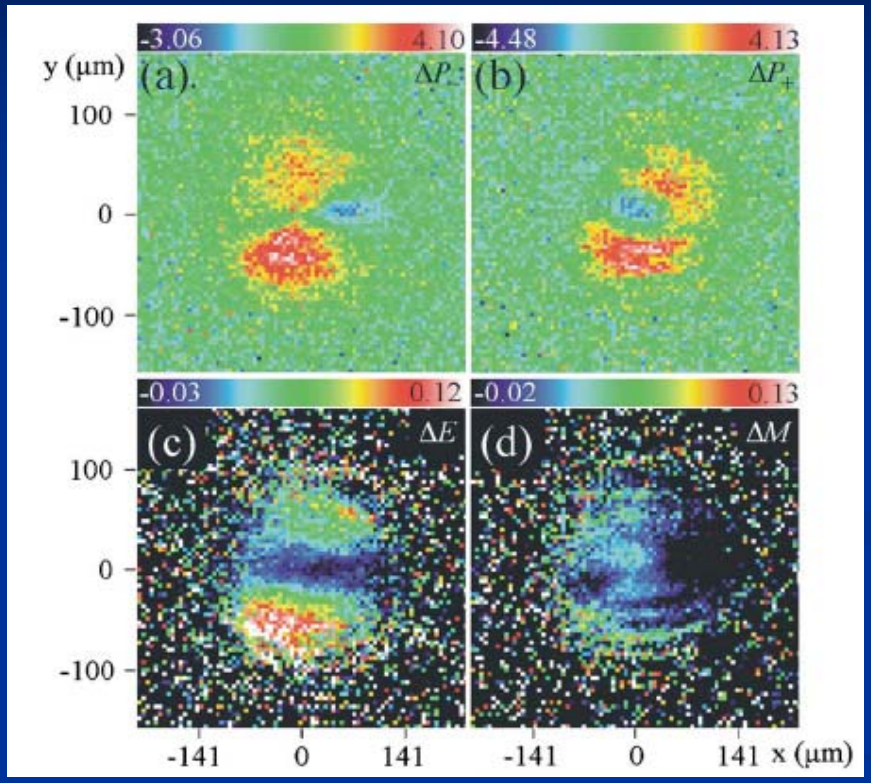
Resolution comparison



- 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

Right circularly polarized



Left circularly polarized

ΔE from gradient of electron density

ΔM (spin polarization) is opposite in +x and –x directions

$P^{(2)}_{RCP} + P^{(2)}_{LCP}$ $\quad$ $P^{(2)}_{RCP} - P^{(2)}_{LCP}$

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

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 ↑
    - → piezoelectric field ↓
    - → bandgap PL ↓ → yellow luminescence ↑
- 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)

1. K. J. Weingarten et. al., *IEEE JQE.* **24,** 198 (1988).

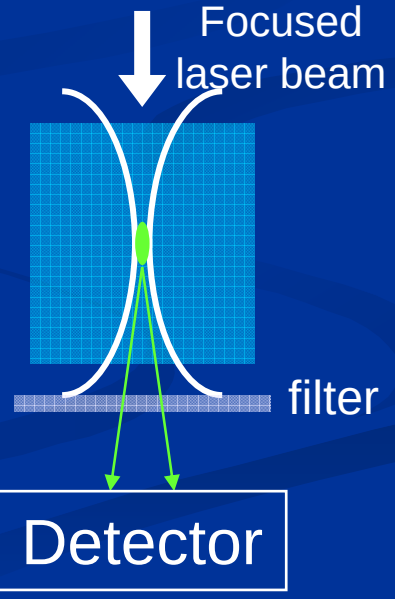
Characteristics of EFISHG

- Electric Field Induced Second Harmonic Generation
  - $P(2\omega) = \varepsilon_0 \chi^3 (2\omega:\omega,\omega,0) E_{laser} E_{laser} E_{applied}$

➔ $I_{EFISHG} \propto (I_{laser})^2$

$I_{EFISHG} \propto (E_{applied})^2 \propto (V_{applied})^2$

- Intrinsic sectioning power ➔ 3D visualization
- Sub-μm resolution
- Ability of measuring electric field vector $\vec{E}$



Visualize E-field by EFISHG

- Surface EFISHG
  - Silicon MMIC[1] & Si/ /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

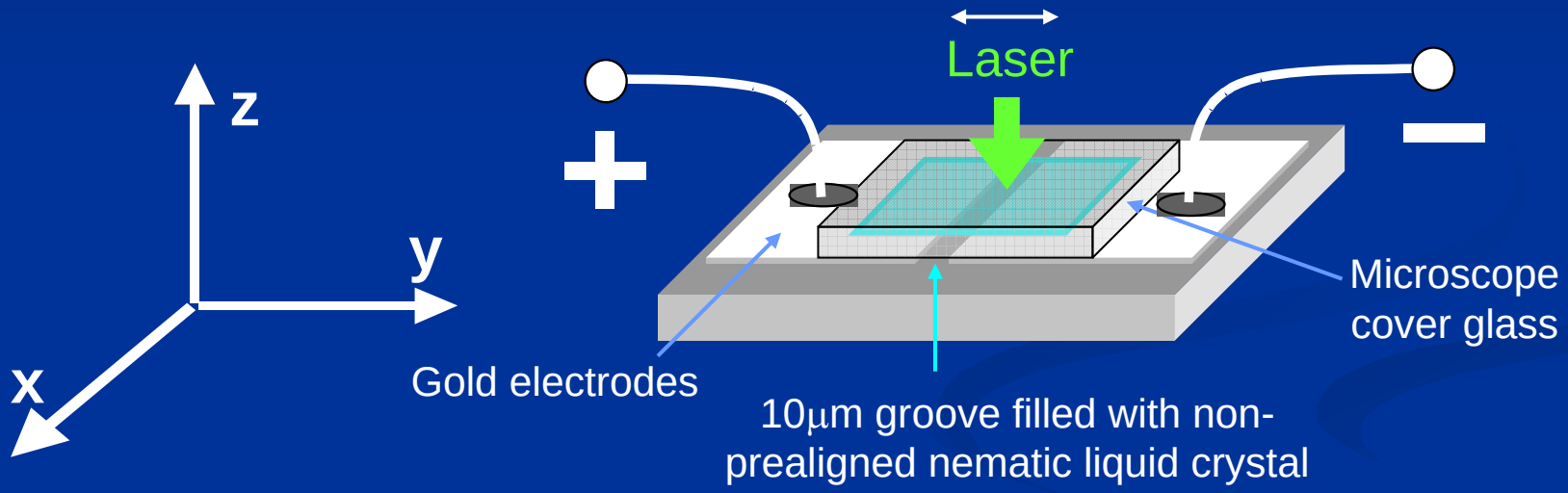
1. C. Ohlhoff, APL **68**, 1699 (1996)
2. J. I. Dadap, PRB **53**, R7607 (1996)
3. C. K. Sun, *APL* **77**, 2331 (2000)

HOM with EFISHG in liquid crystal

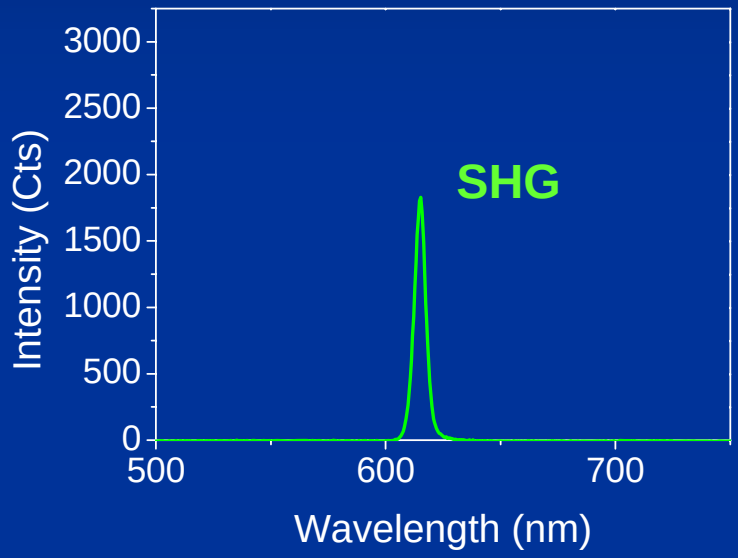
- Advantages:
  - High EFISHG efficiency
  - Background free
  - 3D E-field visualization
  - Measure both amplitude and direction
  - Transparent
  - Non-conducting
  - Easily available

S. K. Saha, *Optics Commun.*, **30**, 119 (1979)

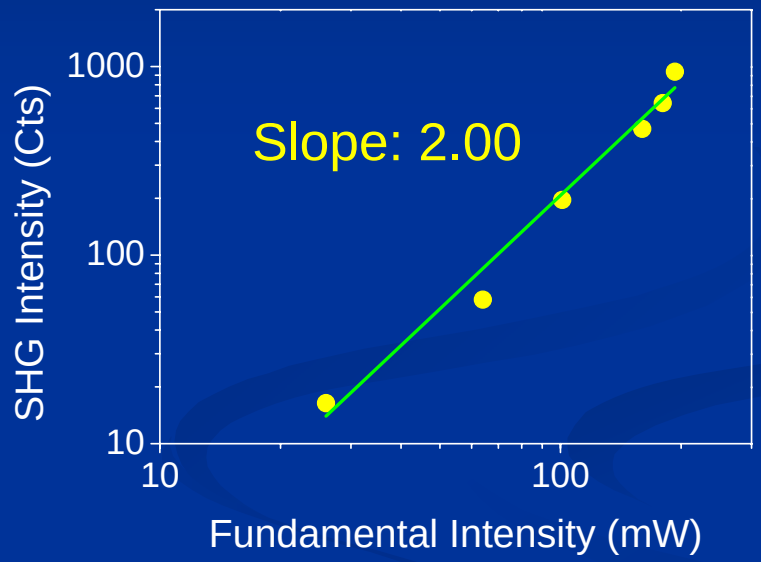
Integrated-Circuit-Like Sample



SHG confirmation

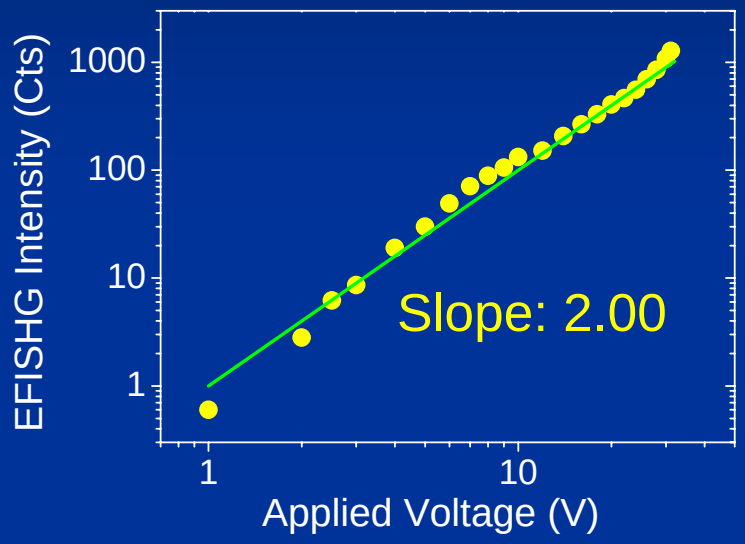


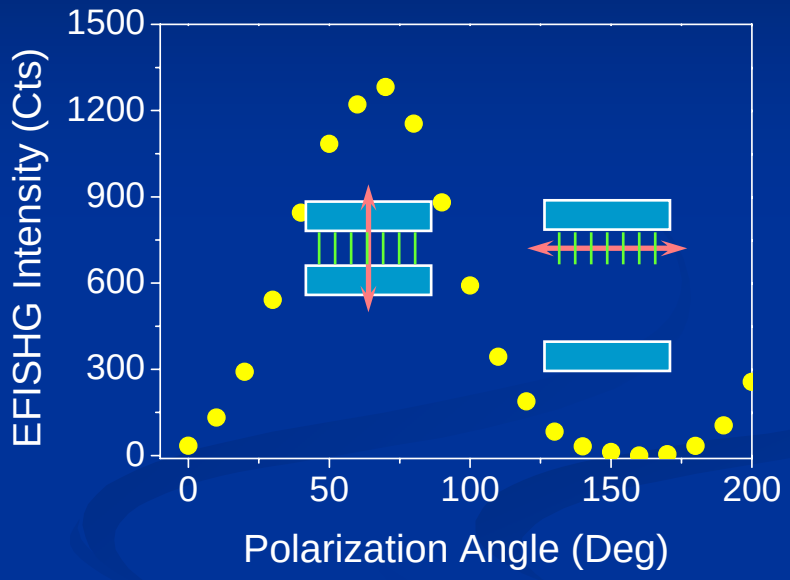
Emission spectrum of liquid crystal (30V)



$I_{EFISHG} \propto (I_{laser})^2$

EFISHG confirmation





$I_{EFISHG} \propto (E_{applied})^2 \propto (V_{applied})^2$

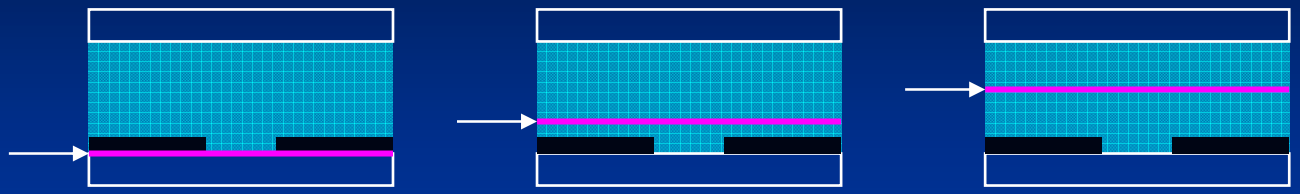
Background free

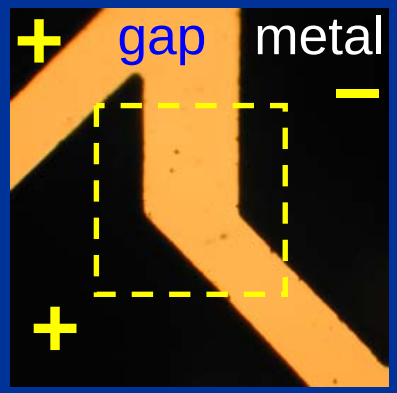
$\chi 3_{xxxx}(\omega,\omega,0) >> \chi 3_{xxxy}(\omega,\omega,0)$

Direction sensitive

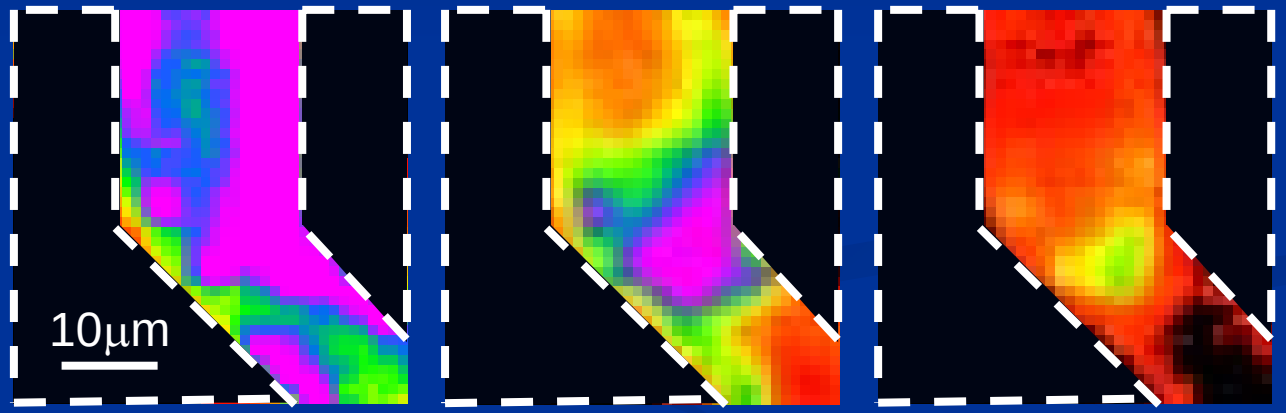
I-H. Chen & S.-W. Chu, *Opt. Lett.* 28, 1338-1340 (2003)

Depth resolution





polarization



In the plane of the electrode

2.5μm higher

5μm higher

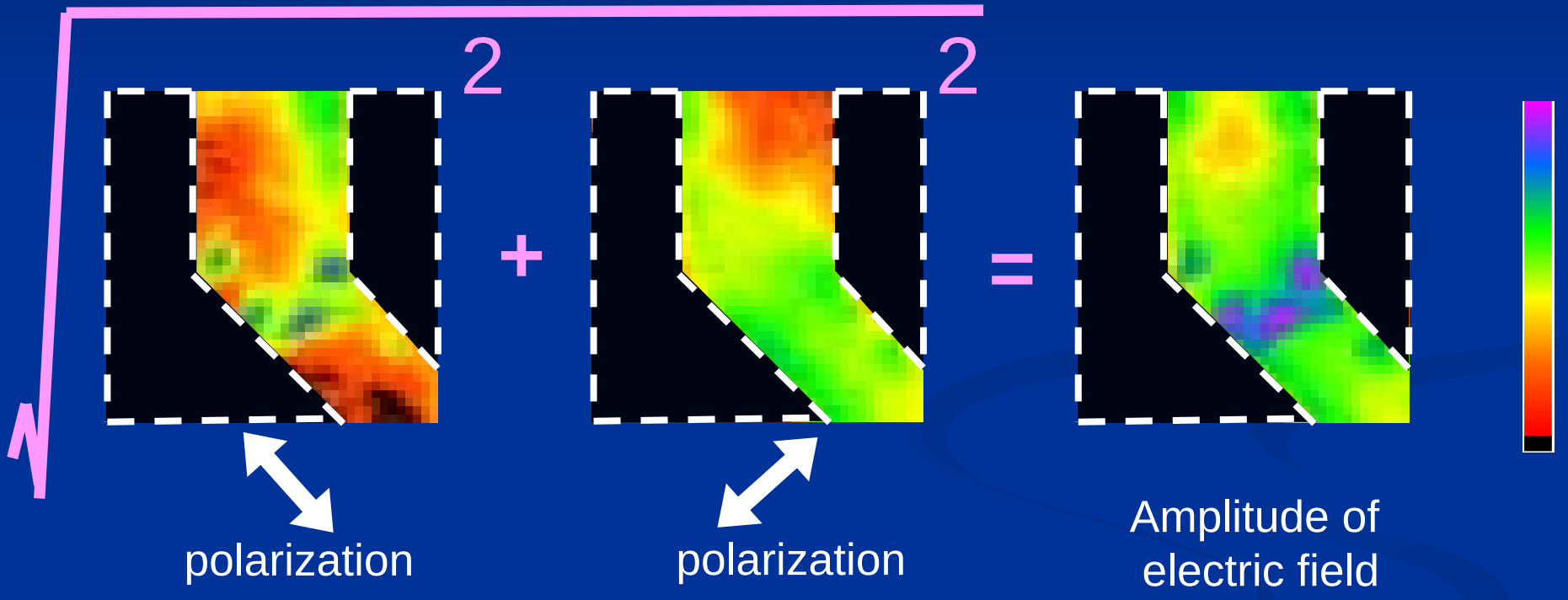
strong

weak

Resolution: xy ~ 0.5μm, z ~ 1μm

E-field visualization

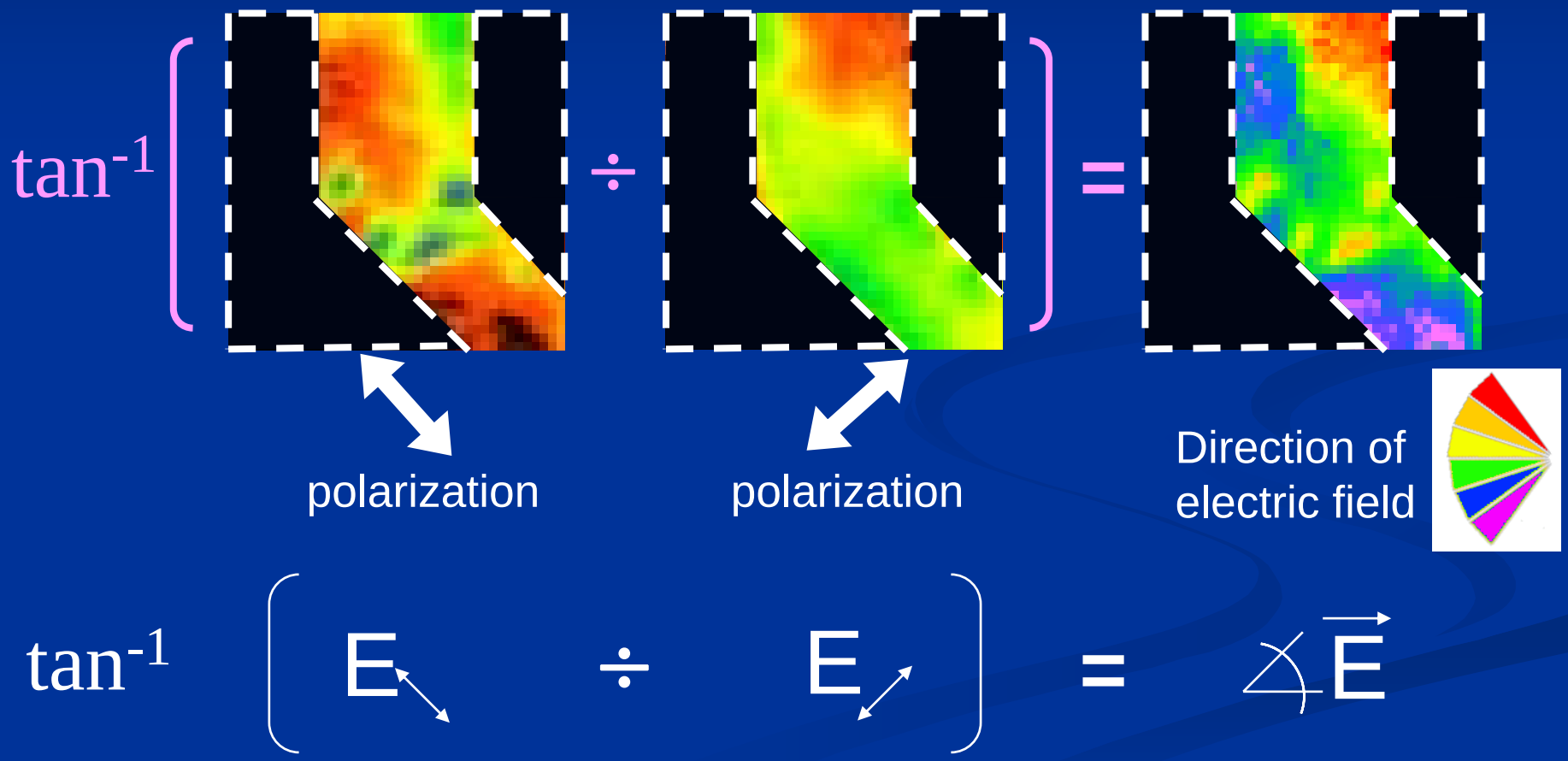
- Amplitude reconstruction



$$\sqrt{E_{\swarrow\nearrow}^2 + E_{\swarrow\nearrow}^2} = |\vec{E}|$$

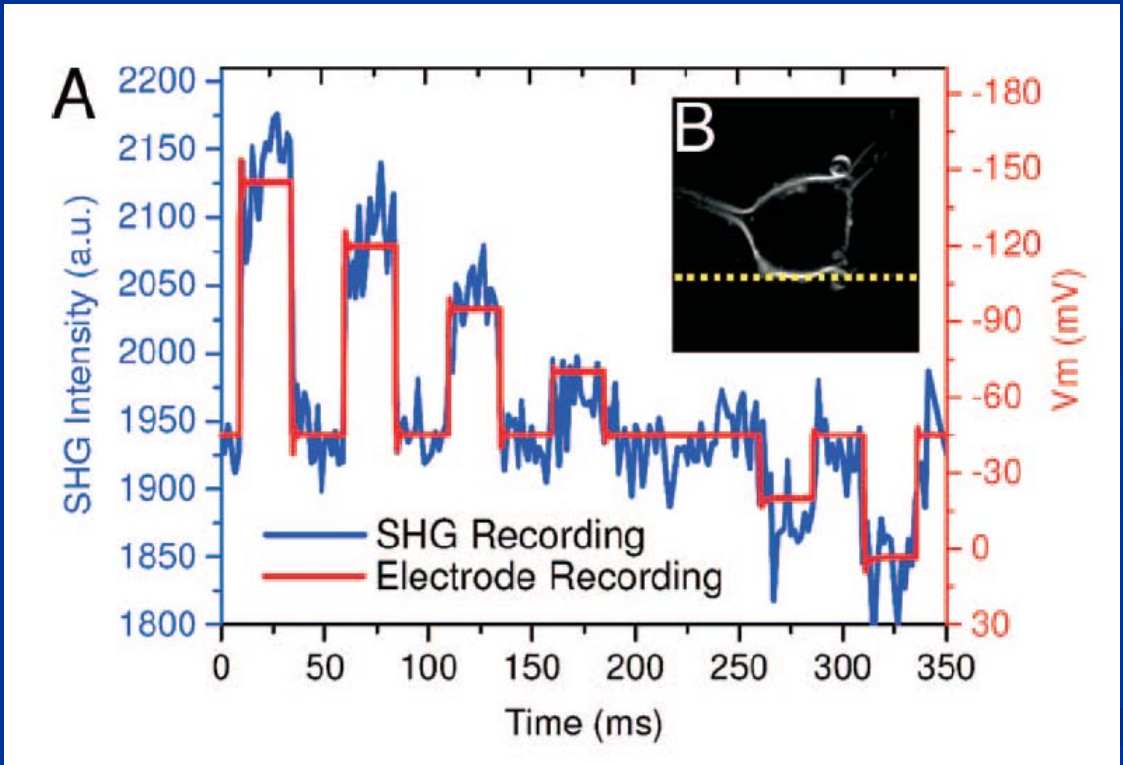
E-field visualization

- Direction reconstruction



Electric field in neuron

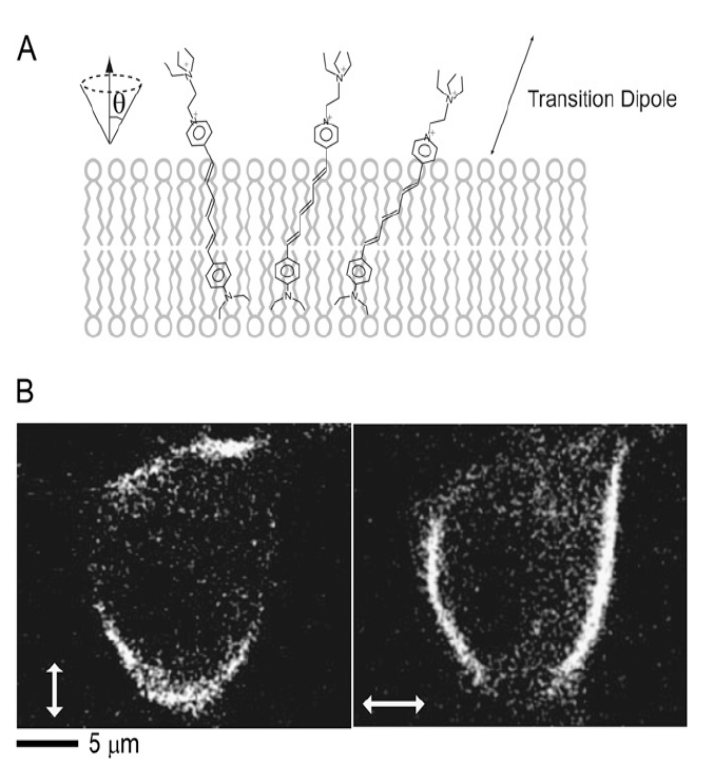
- SHG imaging for neural action potential visualization

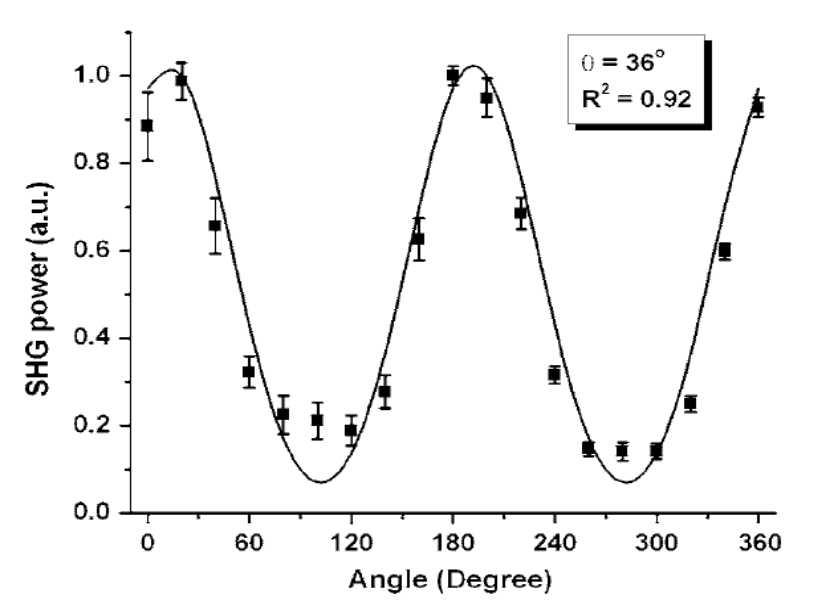


Sacconi, PNAS 103, 3124 (2006)

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

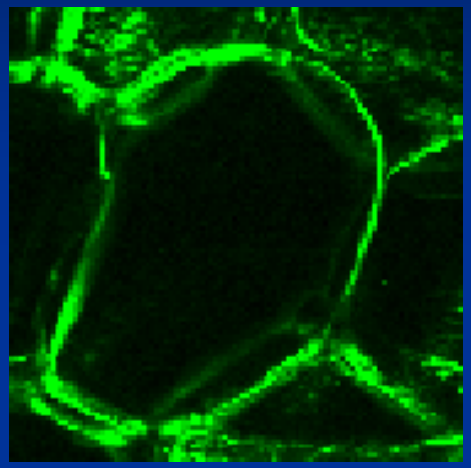
- HOM with EFISHG in LC
  - First 3D E-field visualization
  - Sub-μm spatial resolution
  - Background free
  - Obtain E-field vector
    - Z-component → sample rotation
- Action potential in neuron

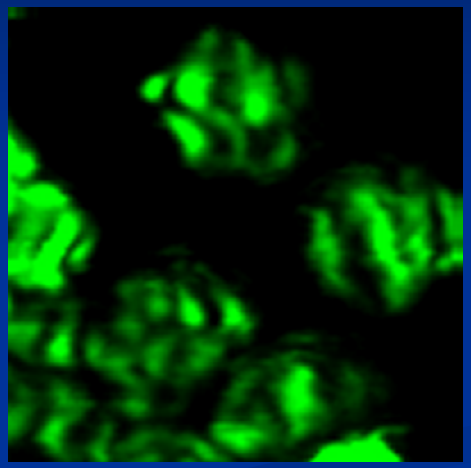
Outline

- 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

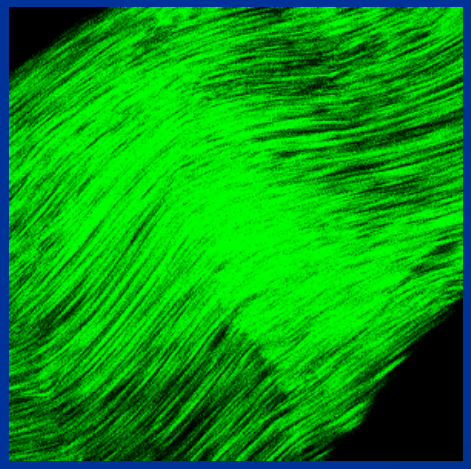
Cellulose in cell wall of maize stem

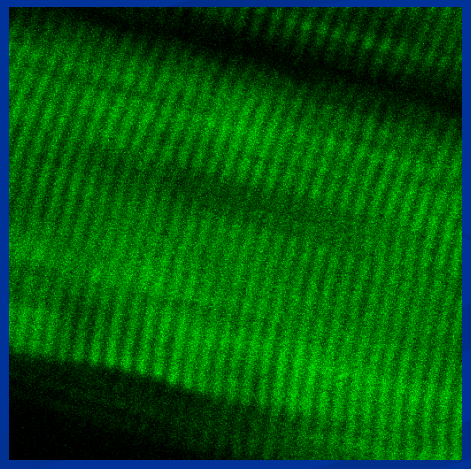




Starch and grana in mesophyll cells

Collagen of tendon fiber





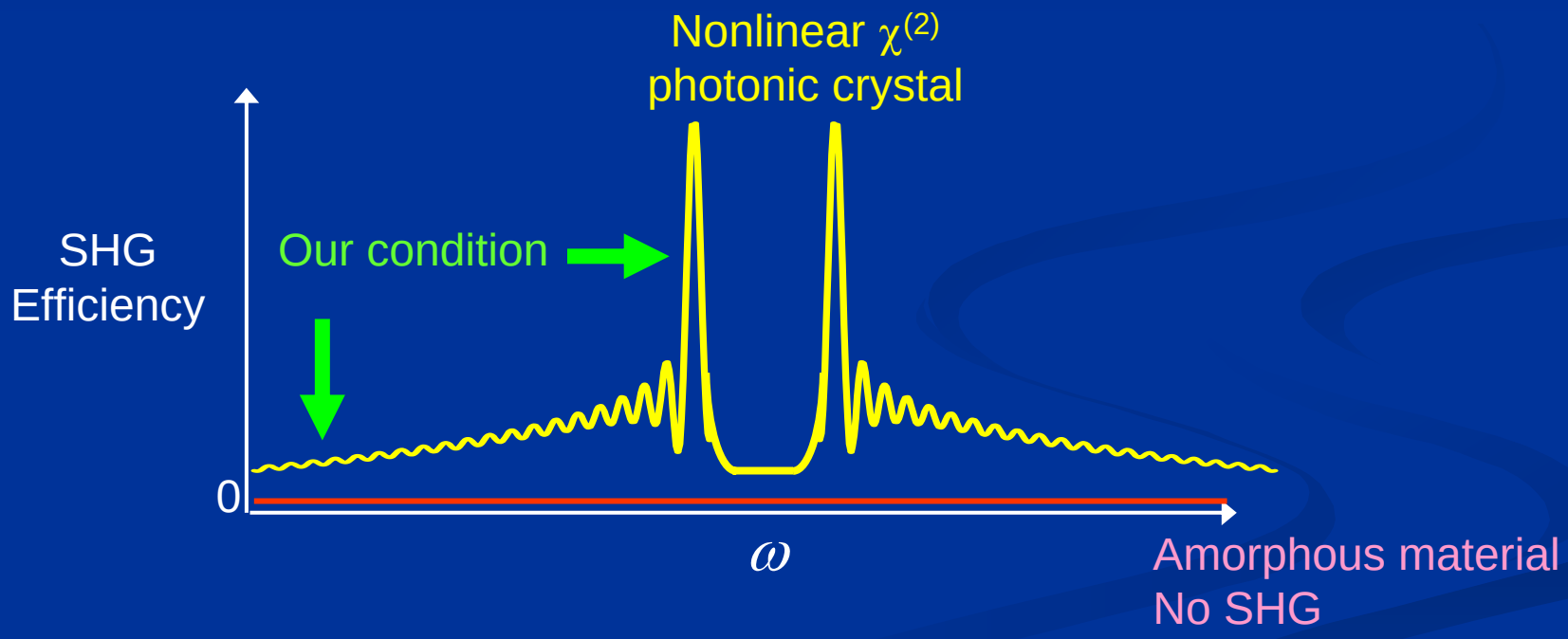
Myosin in muscle fiber

Hint of crystallinity

- No labeling at all!

Nonlinear photonic crystal

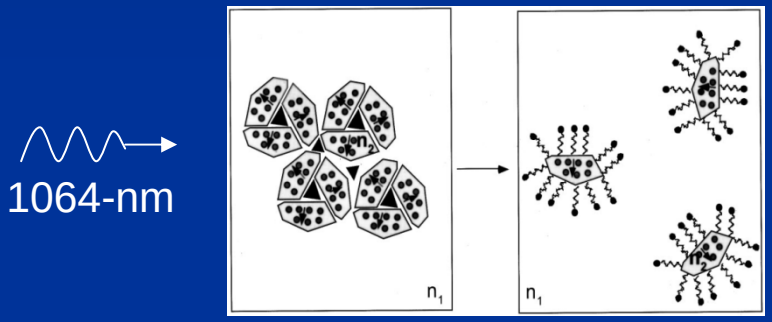
- $\chi^{(2)}$ existed even with pump frequency not close to photonic bandgap.



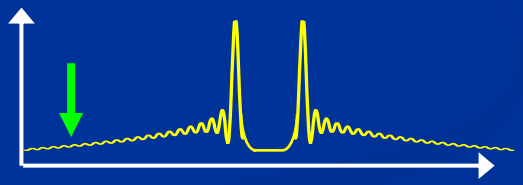
Broderick, PRL 84, 4345 (2000)
Dumeige, APL 78, 3021 (2001)

Nonlinear bio-photonic crystal

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



Bacteriorhodopsin (bR)
on purple membrane

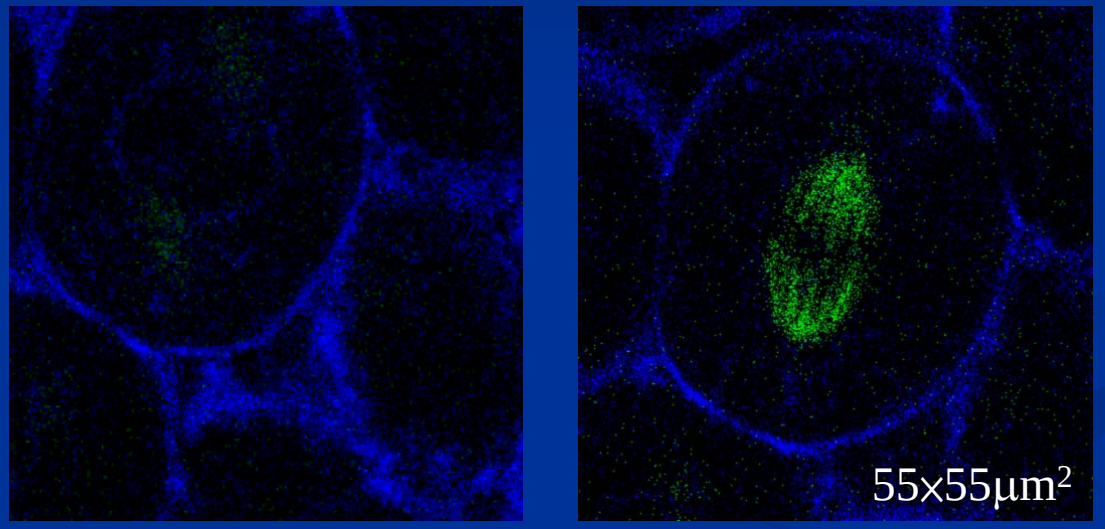


K. Clays, *JOSA B* 18, 1474 (2001).

- 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

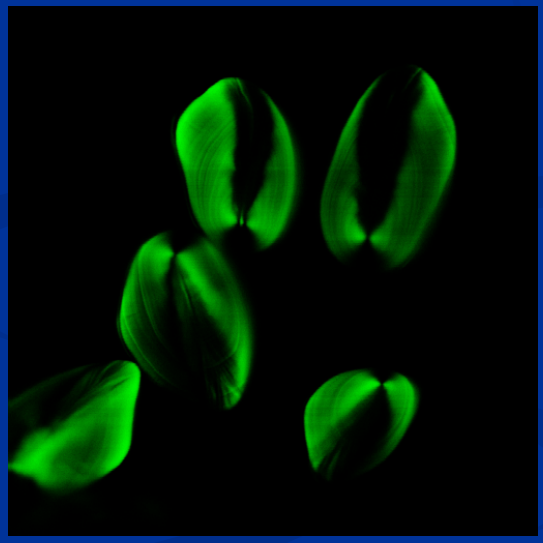
Biophotonic crystal

- Can we find the arrangement symmetry of underlying molecules by SHG?
  - Active molecule identification
  - Molecular structural/packing information elucidated

SHG of starch

Bright field

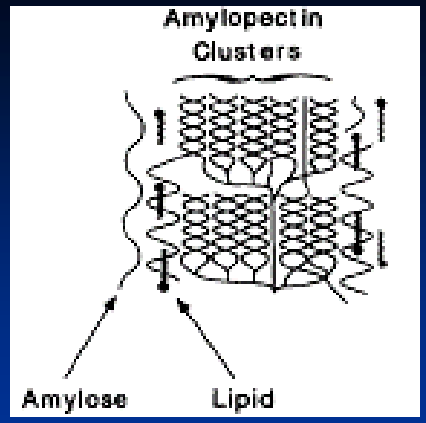
Polarized microscope



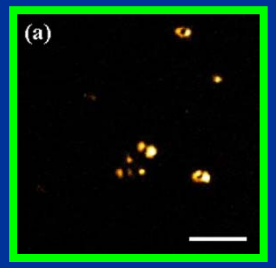
SHG

Molecular origin of starch SHG

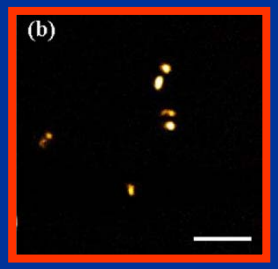
- Amylopectin[1] or amylose[2]?

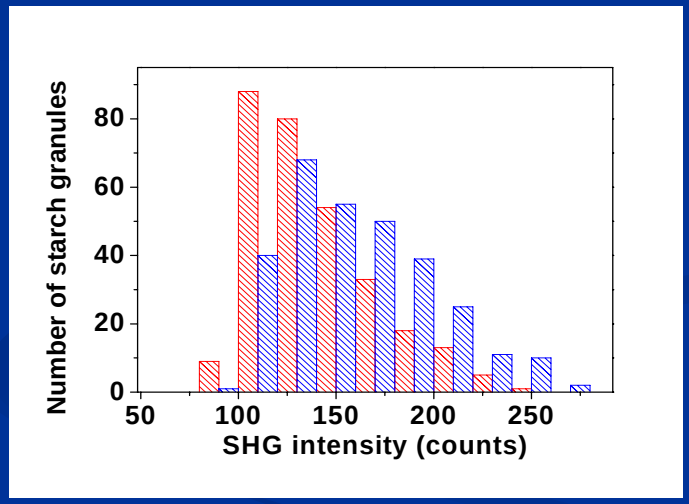


Japonica rice
Amylopectin: 86%
Amylose: 14%



Japonica waxy rice
Amylopectin: 99%
Amylose: 1%





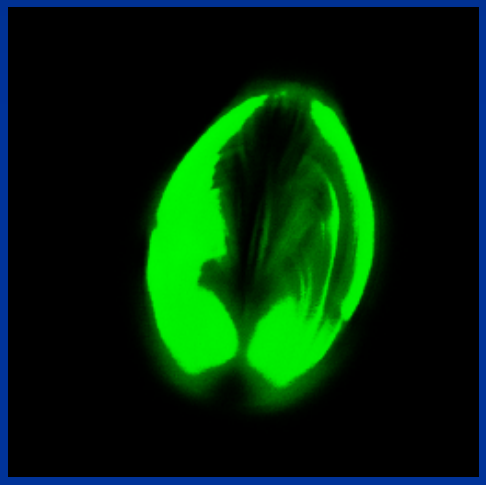
SHG from Japonica waxy rice is 15% stronger

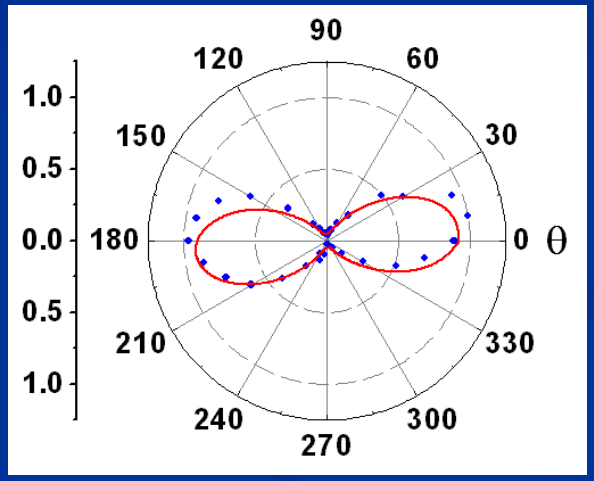
➔ **SHG from amylopectin!!**

1. S.-W. Chu, *J. Microscopy,* **208**, 190 (2002)
2. G. Cox, J. Biomed. Opt. **10**, 024013 (2003)
3. In preparation to Biophys J

SHG of starch

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

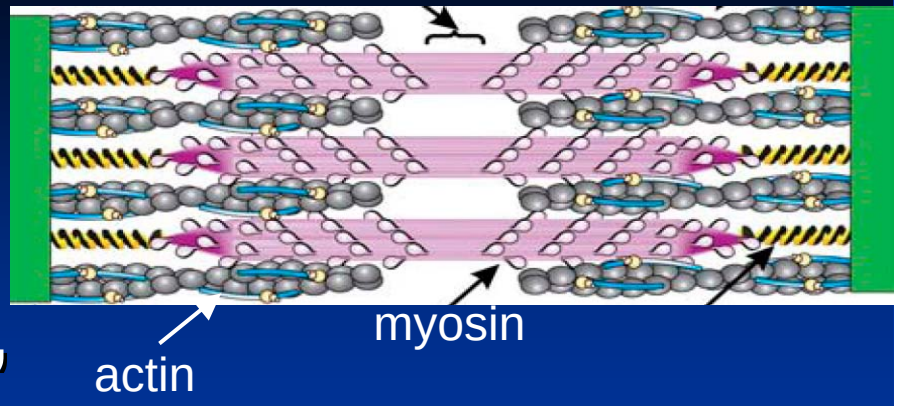




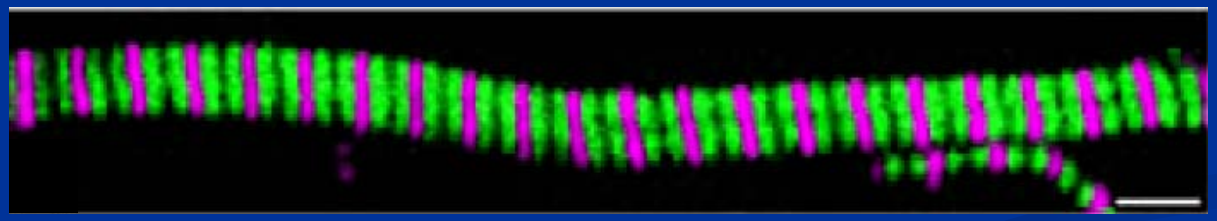
$$SHG \propto \left(\chi_{16}^{(2)} \sin 2\theta\right)^2 + \left(\chi_{21}^{(2)} \sin^2\theta + \chi_{22}^{(2)} \cos^2\theta\right)^2$$

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

SHG in muscle



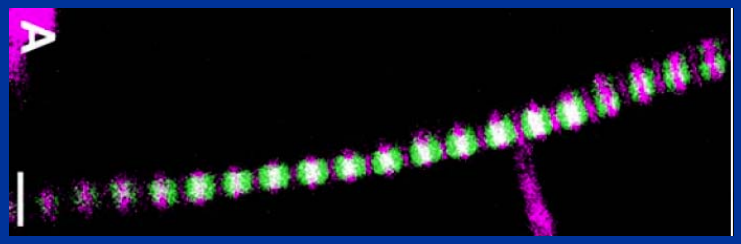
- From myosin filaments, not actin

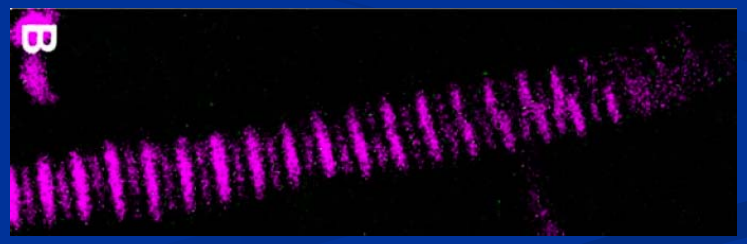


SHG

Actin

Myosin extracted



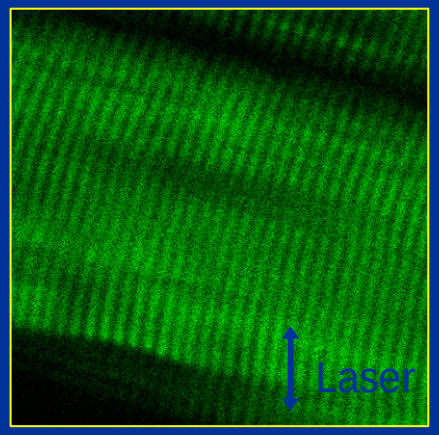


Plotnikov, *Biophys. J.* **90**, 693 (2006)

2D Bio-photonic crystal in animal

Muscle fibers

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

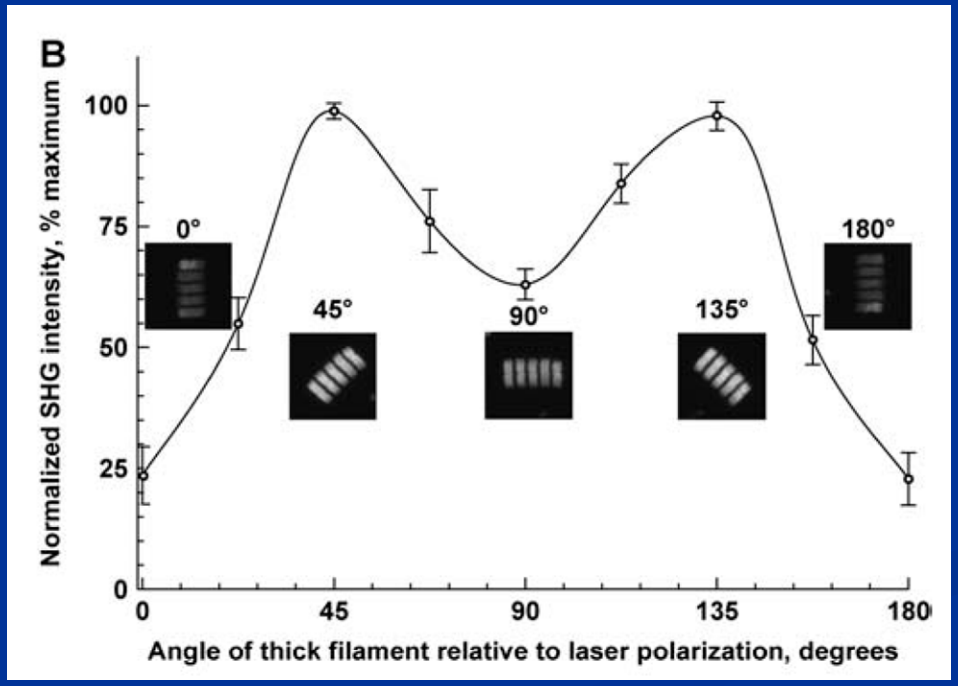


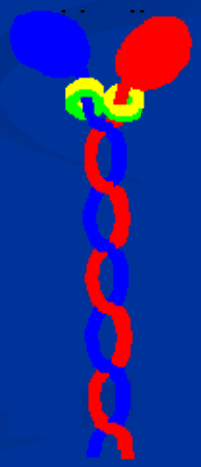
$$\chi^{(2)} = \chi_{31}^{(2)} \begin{bmatrix} 0 & 0 & 0 & 0 & 1.15 & 0 \\ 0 & 0 & 0 & 1.15 & 0 & 0 \\ 1 & 1 & 0.09 & 0 & 0 & 0 \end{bmatrix}$$

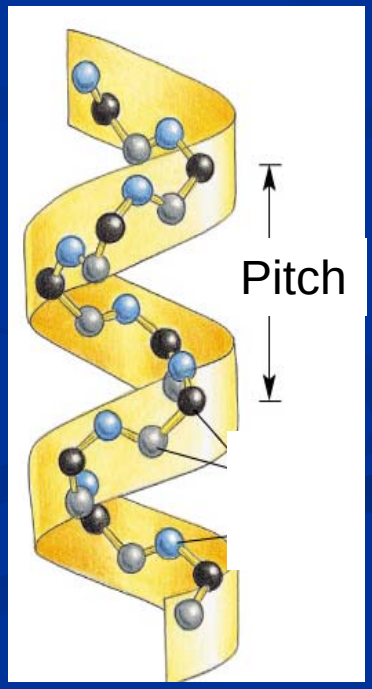
S.-W. Chu, *Biophys. J.* **86**, 1 (2004)

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



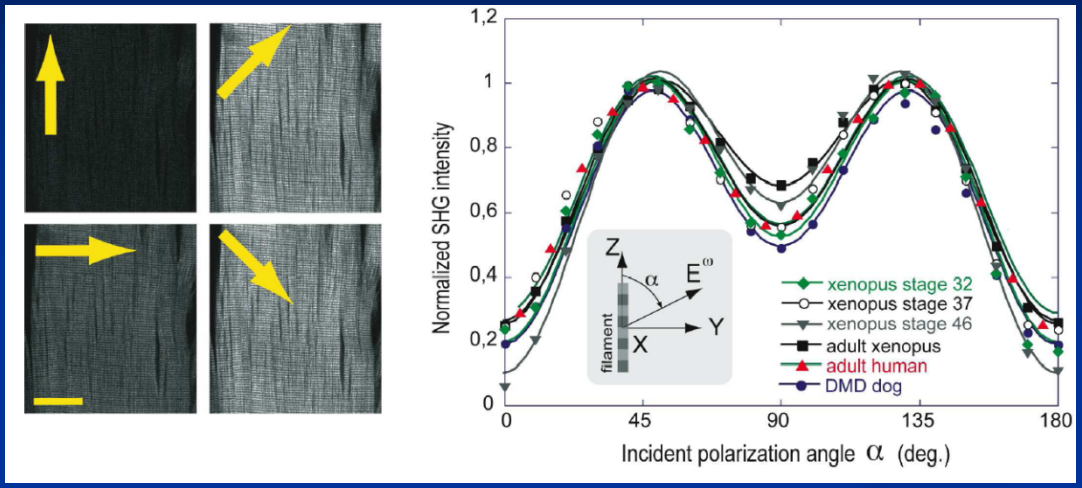




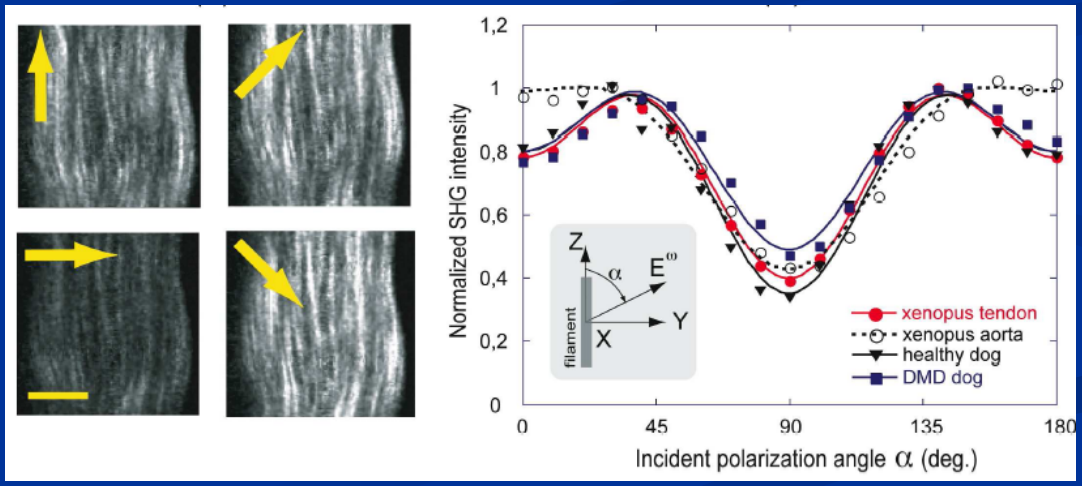
Plotnikov, *Biophys. J.* **90**, 693 (2006)

SHG anisotropy

- Muscle



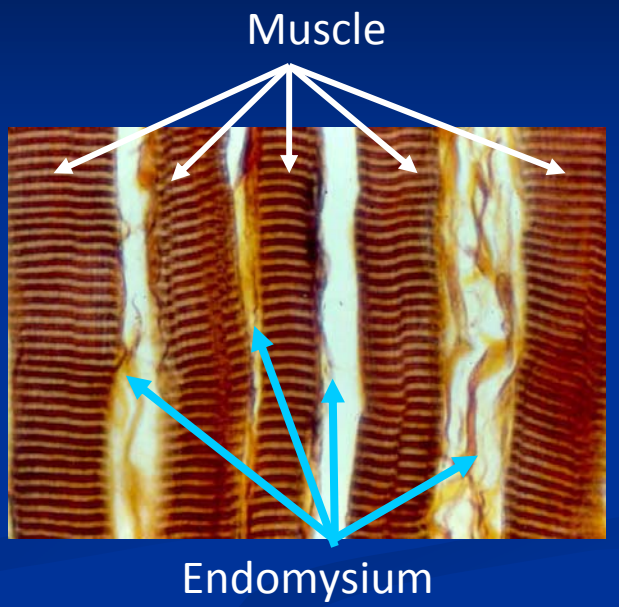
- Collagen

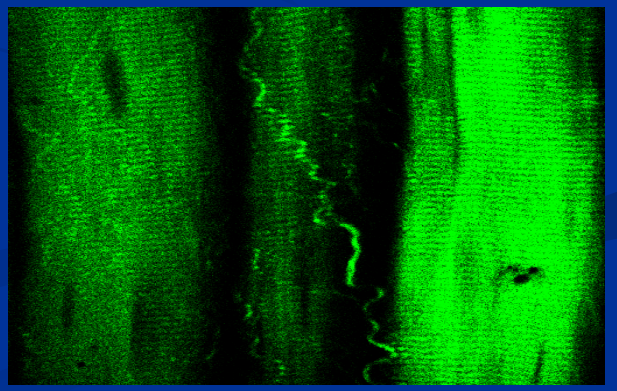


Tiaho, *Opt. Express* **15**, 12286 (2007)

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?

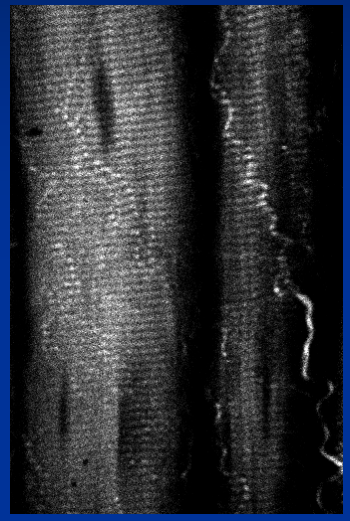


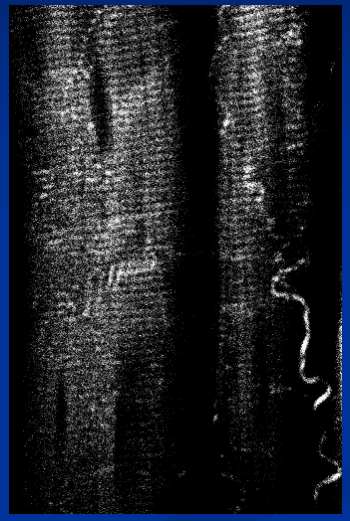


Polarization based selective imaging

Over 100-fold contrast enhancement

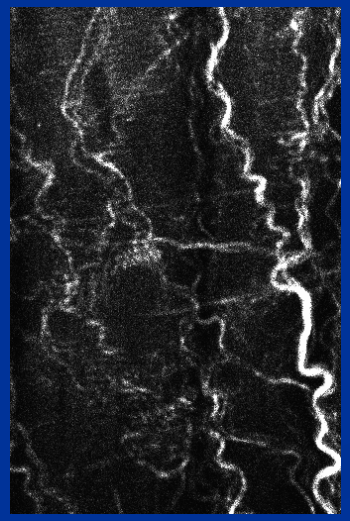
Laser polarization ↔
Polarizer ↔

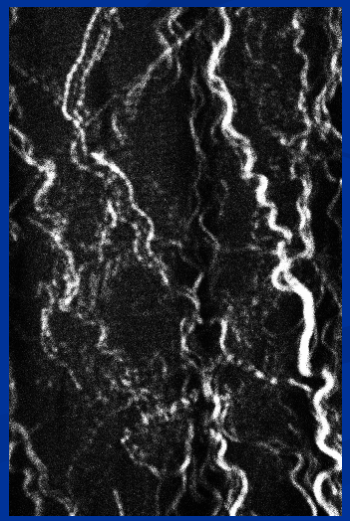




Laser polarization ↔
Polarizer ↕

Laser polarization ↕
Polarizer ↔





Laser polarization ↕
Polarizer ↕

Chu, *APL* 91, 103903 (2007)

Emission dipole based selective imaging

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

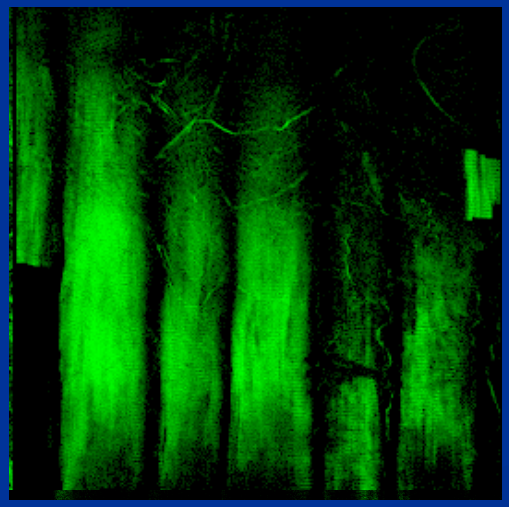
Forward-SHG

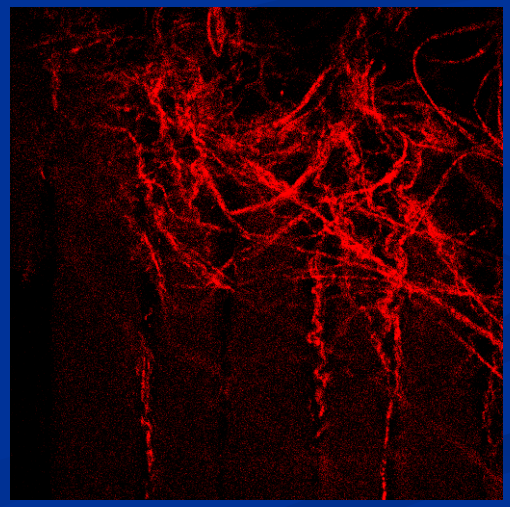
Polarizer ⟷

Backward-SHG

No polarizer

Laser polarization ⟷

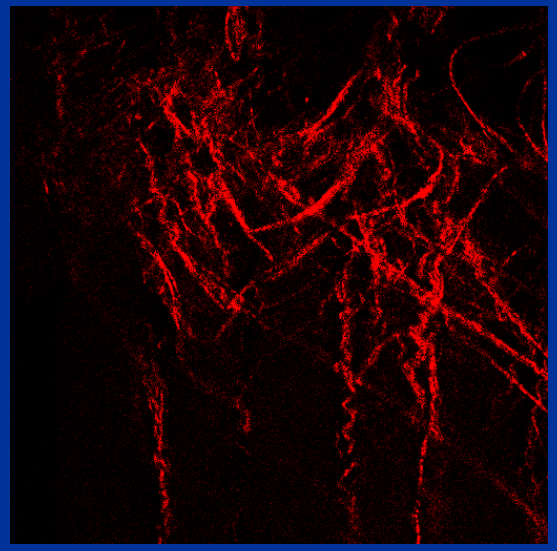




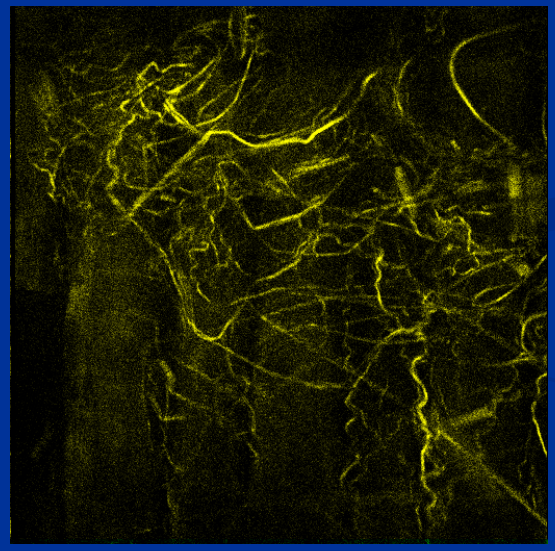
Chu, *J Biomed Opt* 14, 010504, 2009

BSHG vs. FSHG

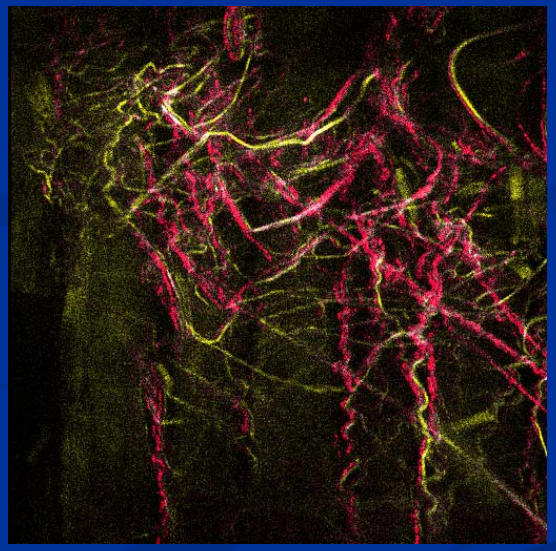
- They do not overlap well
  - BSHG does not come merely from backscattering



BSHG



FSHG

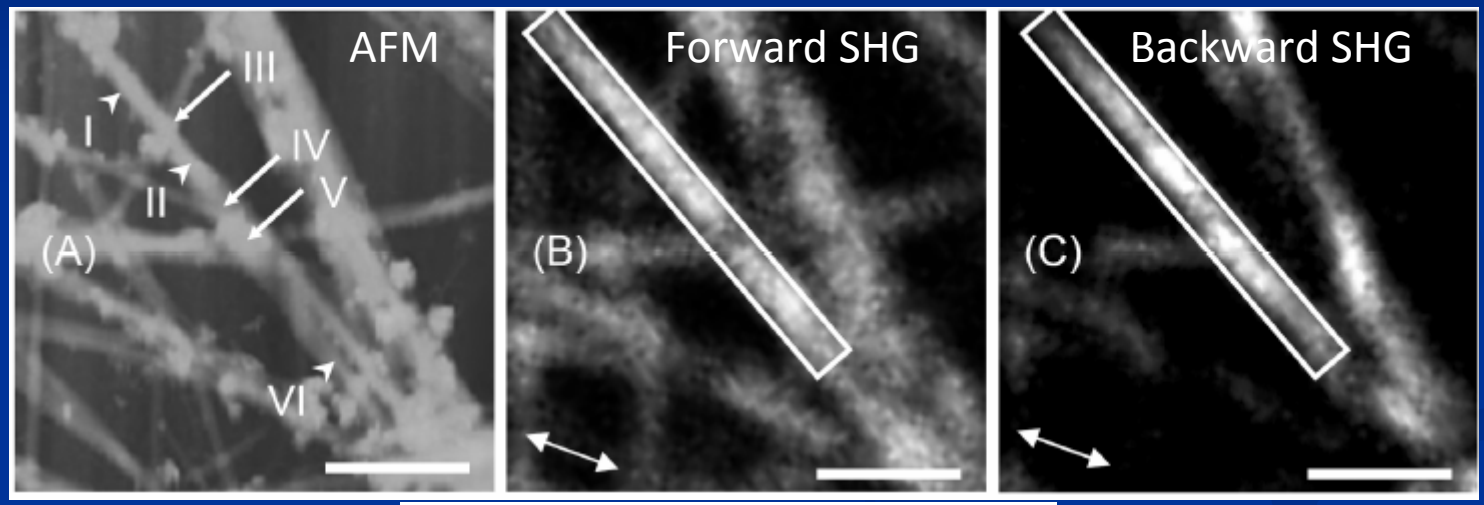


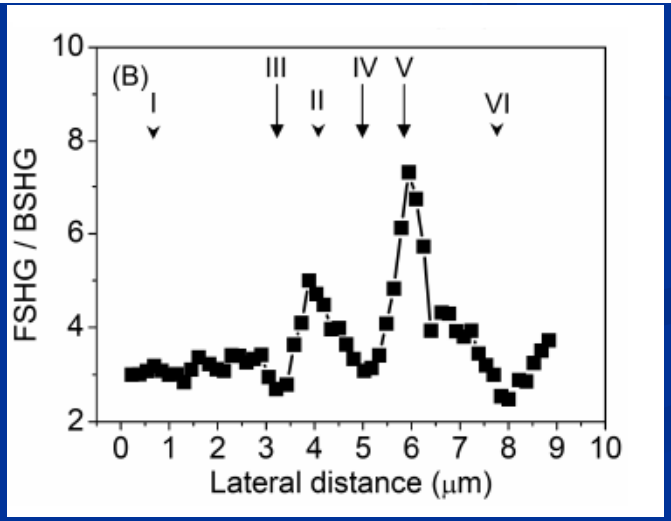
B+FSHG

Laser polarization ↕
Polarizer ↕

BSHG vs. FSHG

- Thickness determination in a collagen fibril

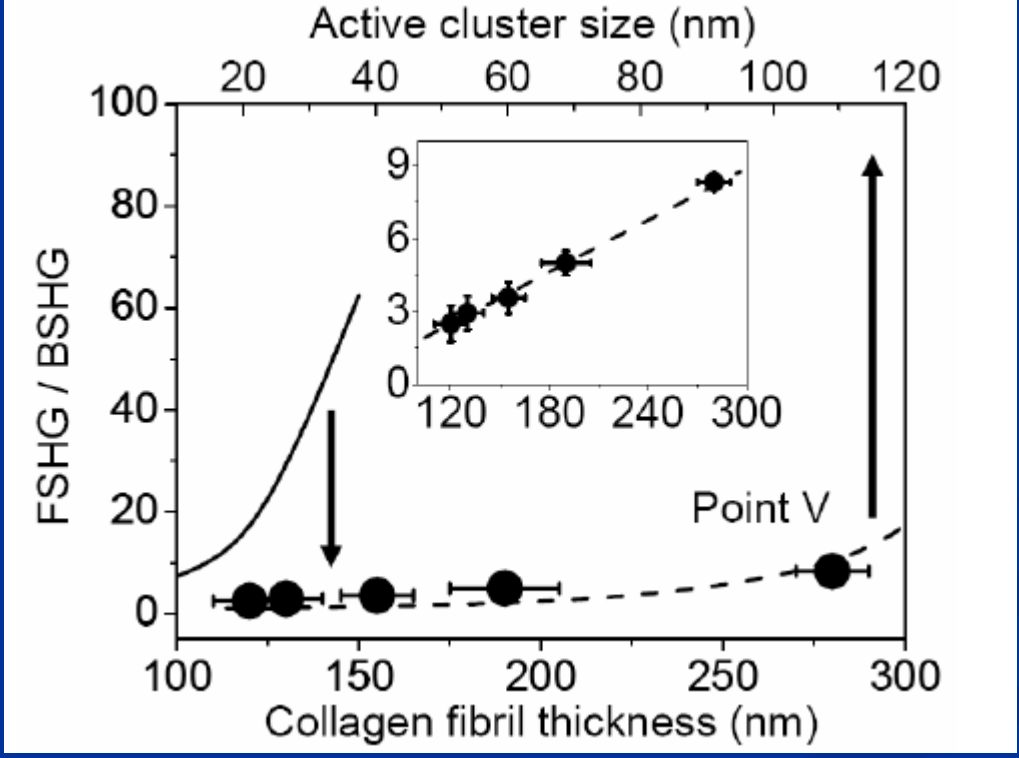




Chu, *Opt. Express*, 15, 12005 (2007)

Thickness of a collagen fibril

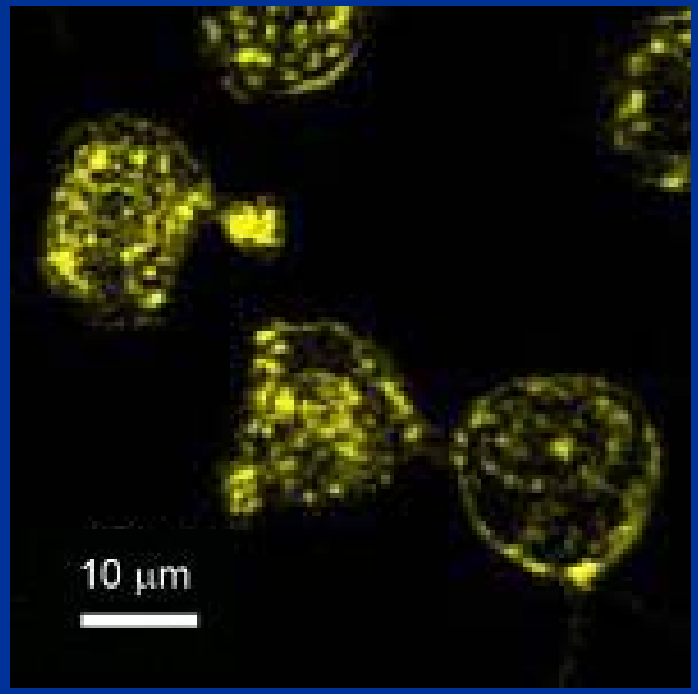
- Determined by FSHG/BSHG ratio
  - Ten nanometer precision



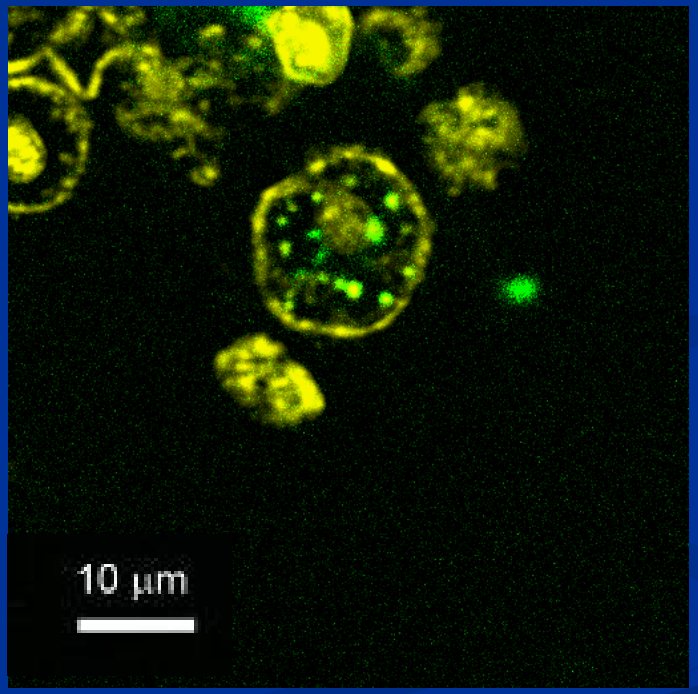
Virus imaging

- No labeling is required

Normal cells



Infected cells



Nuclear polyhedrosis viruses in living cells

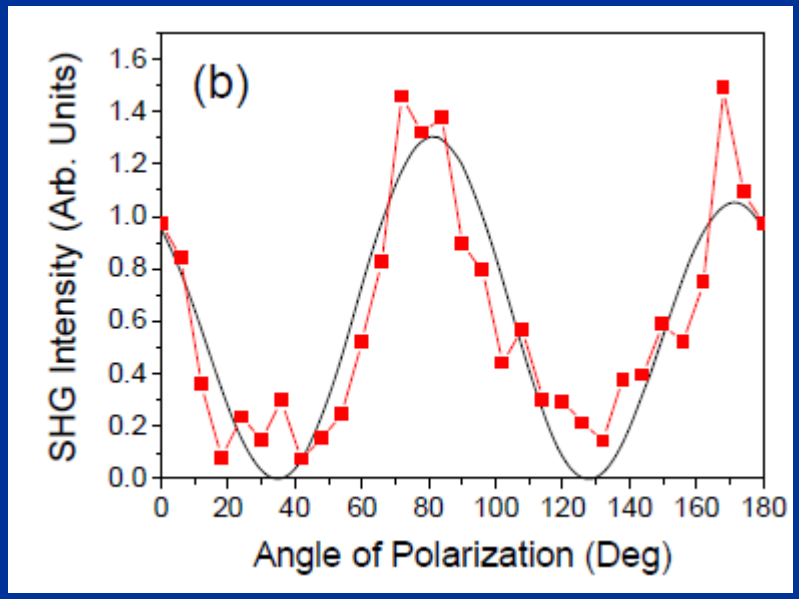
SHG to locate the virus

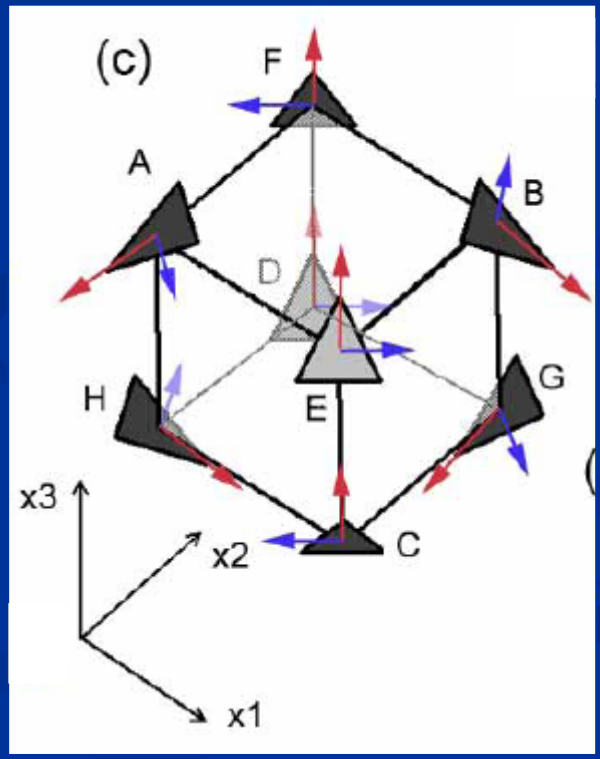
THG to outline the cells

Liu, *Opt. Express* 16, 5602 (2008)

SHG polarimetry

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





Future prospect

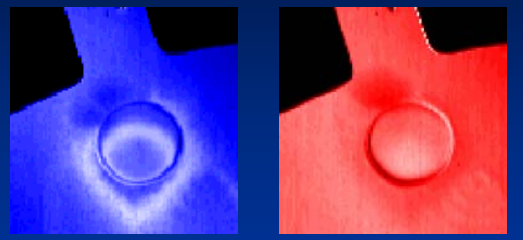
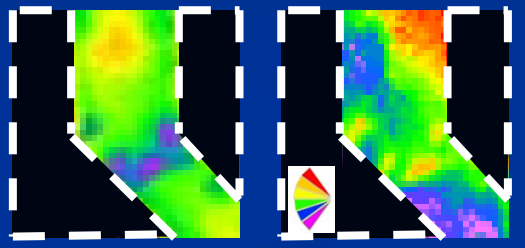
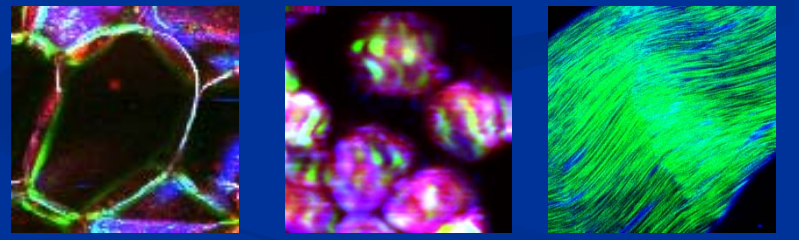
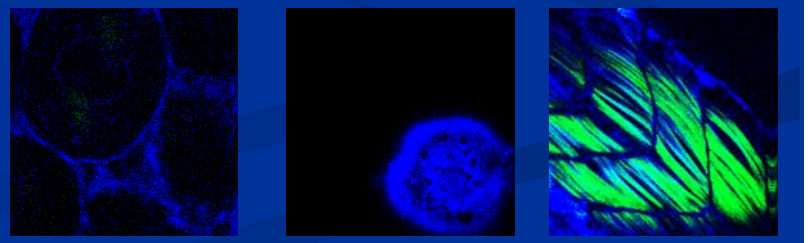
- SHG is sensitive to molecular structure
  - Membrane / thin-film study
  - Spin dynamics mapping
  - Electric field visualization
  - Thermal effect probing
  - Deep tissue imaging

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
  - 陳毅修
  - 陳政吉
  - 陳思妤
- 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!