Nonlinear optical imaging at the nanoscale

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Neurons imaged in 3D with sub-micrometric resolution.
Neuronal responses imaged in real time and in 3D
Neuronal circuitry imaged in real time and in 3D

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Chromatic multiphoton serial microscopy

Supatto, Livet, Beaurepaire, Nat. Comm (2019)
doi.org/10.1038/s41467-019-09552-9

Brain Bow 2 photon image, Jean Livet I. de la vision
Nonlinear optical imaging at the nanoscale

Introduction : motivation
Principles of nonlinear optical microscopy
Polarized nonlinear microscopy
Nonlinear microscopy in depth in complex media
Introduction: motivation

Optical waves in microscopy:
Low depth (~100’s μm – 1mm)
High resolution (~ 200 - 300 nm)
The extra-cellular matrix is made of proteins and fibers (collagen, elastin, ..)


Cell membrane

Fundamental limit of optics: absorption in tissues

$0.7 - 1.2 \, \mu m$ : reduced absorption compared to VIS light
Fundamental limit of optics: scattering in tissues

0.7 – 1.2 \( \mu \text{m} \) : reduced Rayleigh scattering compared to VIS light

\(< \lambda \) Rayleigh Regime
- E.g. particles in the sky
- Strongly wavelength dependent
- Mostly isotropic
Fundamental limit of optics: scattering in tissues

0.7 – 1.2 \( \mu m \): reduced Rayleigh scattering compared to VIS light
But strong Mie scattering at a few 100’s um depth!

\( \mu_s' (\text{Rayleigh}) \)

\( \mu_s' (\text{Mie}) \)

\( \mu_s' (\text{Mie} + \text{Rayleigh}) \)

\( \mu_s' (\text{Mie Scattering}) \)

\( \mu_s' (\text{Mie Scattering, larger particles}) \)

\( \mu_s' (\text{Rayleigh Scattering}) \)

\begin{itemize}
  \item E.g. particles in the sky
  \item Strongly wavelength dependent
  \item Mostly isotropic
\end{itemize}

\begin{itemize}
  \item Cells, water droplets (fog)
  \item Anisotropic: mostly forward scattering
\end{itemize}
Fundamental limit of optics: depth in scattering media

Propagation distance (mm)

Ballistic regime

Increasing photon scattering

Random walk

Biological tissue:

\[ l_t \sim 1\text{mm} \]

\[ l_s \sim 100\mu\text{m} \]
Fluorescence
Fluorescence labels for biology: examples

Chromophores:

TMR

Cy5

Lipid probes:

Chaines carbonées (hydrophobe)

Tête polaire (hydrophilie)

Protein GFP:

“Quantum Dots” (CdSe/ZnS):

Dubertret et al., ESPCI
Alivisatos et al., Berkeley U., USA
Fluorescence labels for biology: genetically modified organisms

2008 Nobel Prize in Chemistry

Osamu Shimomura  Martin Chalfie  Roger Y. Tsien

“For the discovery and development of the Green Fluorescent Protein GFP”
Fluorescence

How many photons can we get out of one molecule?

Fluorescence = Absorption \times Emission
1 photon Fluorescence: 1PF

Absorption probability:

\[ P_{\text{abs}} = \sigma_{\text{abs}} \cdot I \]

Fluorescence quantum yield:

\[ \Phi_f = \frac{k_r}{k_r + k_{nr}} \]

Transition dipole moment:

\[ \mu_{01} = \int \psi_1^*(r) \cdot (-e\hat{r}) \cdot \psi_0(r) \, dr \]

Transition dipole moment
1 photon Fluorescence : 1PF

Fluorescence = Absorption \times Emission

Absorption probability :

\[ P_{\text{abs}} = \sigma_{\text{abs}} \cdot I \]

\[ \sigma_{\text{abs}} \approx 10^{-16} \text{ cm}^2 \]

Fluorescence quantum yield:

\[ \Phi_f = \frac{k_r}{k_r + k_{nr}} \]

\[ \Phi_f \approx 0.5 - 0.9 \]

Absorption probability

\[ I_{1\text{PF}}(t, t') \propto P_{\text{abs}}(t) \]

Emission probability

\[ P_{\text{em}}(t') \cdot e^{-(t-t')/\tau_f} \]
1P absorption rate from a single molecule

Molecule: absorption cross section $\sigma$

Excitation surface: $A$

Absorption rate:

$$P_{abs} = \frac{\langle P(t) \rangle \cdot \sigma}{h \nu A}$$

Typ. $\sim 10^6$ ph/s

Laser $\sim 10^{24}$ ph/s/µm²

Focal spot

$\langle P(t) \rangle$
1 photon fluorescence signal from a single molecule

\[ \langle I(t) \rangle = C \cdot \Phi_f \cdot \frac{\langle P(t) \rangle}{h \nu} \cdot \frac{\sigma}{A} \text{ ph/s} \]

Fluorescence quantum yield:
\[ \Phi_f \approx 0.5 - 0.9 \]

Collection factor:
\[ C \approx 2 - 10\% \]

Fluorescence emission:
\[ \sigma \approx 3 \times 10^{-16} \text{ cm}^2 \]

Raman scattering:
\[ \sigma_{\text{Raman}} \approx 10^{-28} \text{ cm}^2 \]

Volume of excitation (surface: A)
\[ \approx 300 \text{ nm} \]

Excitation:
\[ \langle P(t) \rangle = 10 \mu W \]
\[ A \approx 1 \mu m^2 \]

Fluorescence signal:
\[ \langle I(t) \rangle = 6300 \text{ ph/s} \]

Raman noise:
\[ \langle I \rangle_{\text{Raman}} \approx 10 \text{ ph/s} \]
Local analysis
Spatial localization
Super resolution microscopy

Photophysics: fluctuations due to interaction with environment, orientation changes,

Photobleaching event: reaction with oxygen singulet leads to non fluorescent radical

Molecules interdistance > optical resolution

1 photon fluorescence signal from a single molecule

ex. Cy3 (cyanine) exc. 633nm, em. 670nm
2 photon Fluorescence : 2PF

Fluorescence = 2P Absorption \times Emission

Fluorescence quantum yield:

$$\Phi_f = \frac{k_r}{k_r + k_{nr}}$$

$$\Phi_f \approx 0.5 - 0.9$$

$$I_{2PF}(t, t') \propto \text{P}_{abs}(t) \cdot \text{P}_{em}(t') \cdot e^{-(t-t')/\tau_f}$$

2P Absorption probability \quad Emission probability
Two photon excited fluorescence (2PF) efficiency

1-photon excitation

1 molecule in bright day light: 1 event / s

Absorption probabilities:

\[ P_{abs}^{(1\ photon)} = \left| \mu_{01} \cdot E \right|^2 \]

\[ P_{abs}^{(1\ photon)} = \sigma^{(1)} \cdot E^2 \]

2-photon excitation:

virtual ultra-ultra short lived (<1fs)
intermediate state

1 molecule in bright day light: 1 event / 10 million years

Absorption cross sections:

\[ \sigma^{(1)} \approx (1 - 10) \cdot 10^{-16} \text{ cm}^2 \]

\[ \sigma^{(2)} \approx (10 - 1000) \cdot 10^{-50} \text{ cm}^4 \text{ s/ph} \]

Usual unit: Göppert-Mayer 1 GM = 10^{-50} cm^4 s/ph
Two photon absorption cross section

Intrinsic

Fluorescent proteins (GFP, etc) 10-100 GM

Engineered

Engineered fluorophores with enhanced 2PEF 100-1000 GM

Endogenous fluorophores (NADH, etc) $10^{-3} - 10^{-1}$ GM

Standard fluorophores (Rhodamine...) 0.1-10 GM
2 photon fluorescence signal from a single molecule

\[
\langle I^{2ph}(t) \rangle = C \Phi_f \left( \frac{\langle P(t) \rangle}{h \nu_{2ph} A} \right)^2 \sigma^{(2)} \text{ph/s}
\]

Fluorescence quantum yield: \( \Phi_f \approx 0.5 - 0.9 \)

Collection factor: \( C \approx 2 - 10\% \)

Molecule 2 photon absorption cross section: \( \sigma^{(2)} = 100.10^{-50} \text{cm}^4 \text{s/ph} \)

In the continuous (CW) excitation regime:

\[
\langle P(t) \rangle = 10\text{mW} \quad \text{and} \quad \langle I^{2ph}(t) \rangle = 0.6 \text{ph/s} \quad \text{Signal << noise!}
\]

Volume d’excitation (surface: A)

\(~ 300\text{nm}~\)
Short laser pulses are required for two-photon imaging:

Optimal excitation with minimum average power

\[
I^{2PF} = \sigma_{abs}^{(2)} \cdot |\langle E(t)^2 \rangle|^2
\]

**Continuous laser:** \[P(t) = P_0 = \overline{P}\]

\[I^{(TPEF)} \propto \overline{P}^2 = P_0^2\]

**Pulsed laser:** \[P(t) = P_{peak} \cdot f(t)\]

- Rectangle shape
- Width \(\tau\), frequency \(f\):
  \[P_{peak} = \frac{1}{\tau \cdot f} \overline{P}\]

\[I^{(TPEF)} \propto \overline{P}^2 = \frac{P_0^2}{\tau \cdot f}\]

Typically: \(f = 76\text{MHz}, \tau = 220\text{fs}\), for same average power:

\[
I^{(\text{pulsed})} = 6 \times 10^4 \ I^{(\text{CW})}
\]

Hell et al. 1994
Two-photon fluorescence (2PF) from ensemble of molecules

1 molecule: \( I_{I}^{2-ph} \propto |\mu_{abs}(\Omega) \cdot E^{\omega}|^4 |\mu_{em}(\Omega) \cdot I|^2 \)

N molecules: sum of intensities (incoherent process)

\[
I_{I}^{2-ph} = N \int_{\Omega} |\mu(\Omega) \cdot E|^4 |\mu_{em}(\Omega) \cdot I|^2 f(\Omega) \, d\Omega
\]

\[
I_{I}^{2-ph} = N \cdot \Phi_f \cdot C \cdot \sigma^{(2)} \cdot (I^\omega)^2
\]
Microscopy
Optical resolution of a microscope objective

- Minimize aberrations (chromatic / spherical..) and therefore optimize the quality of an image
- Provide a high numerical aperture to gain in optical resolution

\[ \lambda/(2NA) \sim 200\text{nm} \]

\[ \text{NA} = n \cdot \sin \alpha \]

Ideal illumination/object

Real illumination/image

Point Spread Function (PSF)

Immersion medium
Optical resolution of a microscope objective

Real illumination/image
Point Spread Function (PSF)

« Ideal » illumination/object

$\lambda/(2NA) \sim 200\text{nm}$

Fluorescence

Detergent extracted actin cytoskeleton SEM- Ian Wells, DB Stolz
Two photon excited fluorescence (2PF) imaging

\[ P_{abs}^{(1 \text{ photon})} \propto I \]

\[ V^{(1 \text{ photon})} = \frac{0.7\pi n\lambda^3}{NA^3} \]

\[ P_{abs}^{(2 \text{ photon})} \propto I^2 \]

\[ V^{(2 \text{ photon})} = \frac{8n\lambda^3}{\pi^3 NA^4} \]

Reduced volume by about \[ \sqrt[3]{\frac{1}{2}} \]

1 and 2 photon fluorescence in a solution

\( \lambda = 750 \text{nm}, \ NA \ 1.2 \)

1-photon
0.81 \( \mu \text{m} \times 0.37 \ \mu \text{m} \)

2-photon
0.52 \( \mu \text{m} \times 0.26 \ \mu \text{m} \)
Two photon excited fluorescence (2PF) Scanning microscopy
2PF excitation / radiation in a microscope

\[ I_u^{2PF} = N \left( \int_{NA} \int_{V} \int_{\Omega} |\mathbf{\mu}_{abs}(\Omega, \mathbf{r}) \cdot \mathbf{E}(\mathbf{r})|^4 |\mathbf{E}_{em}(\Omega, \mathbf{k}, \mathbf{r}) \cdot \mathbf{u}|^2 f(\Omega) \, d\Omega \, d\mathbf{r} \, d\mathbf{k} \right) \]
From incoherent 2PF to coherent NLO

Incoherent process: 2PF

Coherent nonlinear processes: SHG-THG, FWM, CARS

- Single molecule detection
- Biological systems are labelled

- In-depth detection in tissues
- No labelling
Nonlinear optics regime

Linear regime

Non Linear regime

\[ x(\omega) = x^{(1)}(\omega) \]

\[ \mathbf{P}(\omega) = \varepsilon_0 \chi^{(1)} \mathbf{E} \]

\[ x(\omega) = x^{(1)}(\omega) + x^{(2)}(\omega) + \ldots \]

\[ \mathbf{P}(\omega) = \varepsilon_0 (\chi^{(1)} \mathbf{E} + \chi^{(2)} \mathbf{E} : \mathbf{E} + \chi^{(3)} \mathbf{E} : \mathbf{E} : \mathbf{E} + \ldots) \]

\( \chi^{(n)} \) non linear susceptibility tensors
Is SHG efficient enough for microscopy imaging?

\[ P^{SHG} = \chi^{(2)}(2\omega; \omega, \omega): E^\omega E^\omega \]

SHG requires non-centrosymmetry
SHG from a molecule to an ensemble

\[ p^{SHG} = \beta(2\omega; \omega, \omega) : E^\omega E^\omega \]

Typically

\[ \beta = 10^{-48} \text{ to } 10^{-38} \text{ m}^4/\text{V} \]

\[ \beta_{zzz}(2\omega; \omega, \omega) = \frac{3 \mu_{01}^z (\mu_{11}^z - \mu_{00}^z)}{2 (\hbar \omega_0)^2} \frac{\omega^4}{(\omega_0^2 - 4\omega^2)(\omega_0^2 - \omega^2)} \]

involves \[ \mu_{01} \sim \langle \psi_0(r) . r . \psi_1(r) \rangle \]
SHG from a molecule to an ensemble

\[ \mathbf{p}^{\text{SHG}} = \beta(2\omega; \omega, \omega) : \mathbf{E}^\omega \mathbf{E}^\omega \]

\[ p_i^{\text{SHG}} = \sum_{jk} \beta_{ijk} E_j^\omega E_k^\omega \]

Typically

\[ \beta = 10^{-48} \text{ to } 10^{-38} \text{ m}^4/\text{V} \]

\[ \beta_{zzz}(2\omega; \omega, \omega), \]
\[ \beta_{zxx}(2\omega; \omega, \omega), \beta_{zyy}(2\omega; \omega, \omega) \]

involves \[ \mathbf{\mu}_0 \sim < \psi_0(\mathbf{r}).\mathbf{r}.\psi_n(\mathbf{r}) > \]
SHG from a molecule to an ensemble

$\mathbf{p}^{\text{SHG}} = \beta(2\omega; \omega, \omega) : \mathbf{E}^\omega \mathbf{E}^\omega$

$p^{\text{SHG}}_I(\Omega) = \sum_{JK} \beta_{IJK}(\Omega) E_J^\omega E_K^\omega$

Single molecule response

Ensemble response: coherent in-phase oscillation

$p^{\text{SHG}}_I = N \int_\Omega p^{\text{SHG}}_I f(\Omega) \, d\Omega$

$I^{\text{SHG}}_I = |p^{\text{SHG}}_I|^2 = \left| \sum_{JK} \chi^{(2)}_{IJK} E_J^\omega E_K^\omega \right|^2$

$I^{\text{SHG}}_I = N^2 \cdot C \cdot \sigma^{\text{SHG}} \cdot (I^\omega)^2$

N molecules: sum of dipoles/radiated fields (coherent process)
Nonlinear coherent effects: efficiency?

\[ I_{I}^{2-ph} = N \cdot \Phi_f \cdot C \cdot \sigma^{(2)} \cdot (I_\omega)^2 \]

\[ I_{I}^{SHG} = N^2 \cdot C \cdot \sigma^{SHG} \cdot (I_\omega)^2 \]

\[ \sigma^{2PA} \approx 10^{-49} \text{ cm}^4 \text{s photon}^{-1} \]

\[ \sigma^{SHG} \approx 10^{-53} \text{ cm}^4 \text{s photon}^{-1} \]

1 molecule: 1000 – 10000 ph/s

1 molecule: 0.0001 – 0.01 ph/s

10 nm molecular nanocrystal \( \sim \) 1000 dipoles

\[ \beta \sim 10^{-38} \text{ m}^4/V \]

\[ I^\infty \sim 10^{24} \text{ ph/s/cm}^2 \]

S. Brasselet et al. PRL (2004)
SHG and THG Microscopy imaging
SHG excitation / radiation in a microscope

Coherent addition:

\[ I_u^{SHG} = \left| N \int_{\Omega} \int_V \int_{\mathbf{k}} E_u^{SHG}(\Omega, \mathbf{r}, \mathbf{k}) f(\Omega) \, d\Omega \, d\mathbf{r} \, d\mathbf{k} \right|^2 \]
Phase matching effects in SHG imaging

$L$: Dimension of the focal volume

Propagation towards detector or microscope objective

\[ \vec{R}_n \\
\vec{O} \rightarrow \vec{M} \]

\[ E_n^{2\omega} \left( t - \frac{R_n}{c} \right) \]

\[
I^{SHG} \propto |\beta : E_0 \vec{E}_0|^2 \sum_{n,n'} \exp \left( i(2k_\omega - k_{2\omega}) \cdot (r_n - r_{n'}) \right)
\]

\[ \Delta k = k_{2\omega} - 2k_\omega \]

Phase matching wave vector
SHG phase matching under tight focusing:

\[ \Delta k = \Delta k^{\text{SHG}} + \Delta k^{\text{Gouy}} \]

Aprox. : radiation in vacuum

Forward

\[ \left| \Delta k^{\text{SHG}} \right| = 0 + \frac{2.\pi}{2\lambda} \]

Fwd efficient for

\[ r < 2\lambda \sim 2\mu m \]

Object of dimension « r »

Epi

\[ \left| \Delta k^{\text{SHG}} \right| = \frac{8\pi}{\lambda} + \frac{2.\pi}{2\lambda} = \frac{9\pi}{\lambda} \]

Epi vanishes if

\[ r > \frac{\lambda}{4} \approx 250\text{nm} \]
SHG imaging in biological molecules (collagen)

Collagen (EM)

Collagen I is non-centrosymmetric

SHG fwd image of collagen in a muscle tissue
SHG imaging in biological molecules (collagen)
Consequence of phase matching

EPI SHG image  FWD SHG image

Lateral projection (X,Y)
Axial projection (Z,X)

scale bar, 5 μm

Collagen gel (10-μm-thick) SHG imaging
Second Harmonic Generation (SHG) imaging in tissues

Imaging the collagen structure in the tissues thanks to backscattering

Collagen in the stroma

Artificial collagen gel
Third Harmonic Generation (THG) imaging

\[ P^{THG}(3\omega) = \chi^{(3)}(3\omega; \omega, \omega, \omega): E^{\omega} E^{\omega} E^{\omega} \]

Low efficiency
No symmetry condition
Stringent phase matching conditions
THG sensitive to interfaces

Detection of lipid bodies in the drosophila embryo

Intrinsic THG from lipid bodies Exc. 1.180um

E. Beaurepaire, LOB, Palaiseau, France
W. Supatto et al., PNAS (2005)
Coherent Anti-Stokes Raman Scattering (CARS)

\[ P^{CARS}(\omega_{AS}) = \chi^{(3)}(\omega_{AS}; \omega_p, \omega_p, -\omega_s): E^{\omega_p} E^{\omega_p} E^{\omega_s*} \]

Generated frequency by frequency mixing:

\[ \omega_{as} = \omega_p + \omega_p - \omega_s = 2\omega_p - \omega_s \]

Third order process

\[ \chi^{(3)} = \frac{A_R}{\Omega_R - (\omega_p - \omega_s) + i\Gamma_R} + \chi^{(3)}_{NR} \]

Strong signal from a Raman – active band

- 3.1\,\mu m, 99 \,GHz
- 2.9\,\mu m, 102 \,GHz
- 6.6\,\mu m, 45 \,GHz

if \( \omega_p - \omega_s = \Omega \): \( \omega_{as} \) enhanced
Coherent Anti-Stokes Raman Scattering (CARS)

\[ P_{\text{CARS}}(\omega_{\text{AS}}) = \chi^{(3)}(\omega_{\text{AS}}; \omega_p, \omega_p, -\omega_s) : E^\omega_p E^\omega_p E^{-\omega_s} \]

Generated frequency by frequency mixing:

\[ \omega_{\text{as}} = \omega_p + \omega_p - \omega_s = 2\omega_p - \omega_s \]

Third order process

\[ \chi^{(3)} = \frac{A_R}{\Omega_R - (\omega_p - \omega_s) + i\Gamma_R} + \chi^{(3)}_{\text{NR}} \]

Strong signal from a Raman – active band

- 3.1\,\mu m (99 GHz)
- 2.9\,\mu m (102 GHz)
- 6.6\,\mu m (45 GHz)
Coherent Anti-Stokes Raman Scattering (CARS)

\[ \chi^{(3)} = \frac{A_R}{\Omega_{R} - (\omega_p - \omega_s) + i\Gamma_{R}} + \chi^{(3)}_{NR} \]

Coherent superposition of two contributions

CARS is a resonant process

\[ I_{CARS} = \left| \chi^{(3)}_R(\omega_{as}) : E_p E_p E_s^* + \chi^{(3)}_{NR}(\omega_{as}) : E_p E_p E_S^* \right|^2 \]

Polystyrene spontaneous Raman spectrum

Polystyrene CARS spectrum

CARS resonance

Off-resonance

Source: http://www.aist.go.jp
Coherent Anti-Stokes Raman Scattering (CARS)
microscopy

Brustlein et al., JBO 2011
Multimodal imaging for cancer detection
Rat mammary tumor / S. Boppart lab

CARS (3050 cm$^{-1}$)
AutoFluo2P
AutoFluo 3P
SHG
THG

Tu et al. Sc. Adv. 2017
Multimodal imaging for neurosciences

Mouse spinal cord; Collab. F. Debarbieux INT Marseille

Myelin (SEM)

Myelin imaging in the mouse spinal cord

Collaboration F. Debarbieux (INT, Marseille, France)

Myelin (CARS – CH2 stretch. vib.) Axons (TPEF - CFP)

Progression of the EAE disease
CARS imaging in fixed mouse spinal cords: depth 30-50 µm

Myelin (CARS, 2845 cm⁻¹ CH₂)  Axons (2PF, CFP)

Immune cells (YFP, GFP)

P. Gasecka et al., Biophys. J. (2017)
Short pulses and nonlinear microscopy
At 800nm:
Fused silica: GVD = 36.11 fs²/mm
BK7: GVD = 50.60 fs²/mm

A microscope objective: typically 3000 fs²

A diagram showing the relationship between initial and final pulse durations.
Short pulses and nonlinear microscopy

Spectral resolution of the shaper: 0.53nm/px
Max time shift: 1.9ps
Max GVD: 5000fs$^2$

(sub-10fs pulses, M Pawlowska et al. OE 2014)
Short pulses and nonlinear microscopy

Distorsion occurs both in space and time (space-time coupling)

Nanoscatterers:
gold nanorods (34 nm x 25 nm)

After pulse shaping optimization

M Pawlowska et al. OE 22 (2014)
Optimizing spectral conditions in 2P processes

coherent control for selective nonlinear microscopy
Two-photon excitation process

2nd Order Time-Dependent Perturbation Analysis

2P excitation:

\[ a_f(\infty) \propto \int E^2(t) \exp(i\omega_{fg} t) dt \]

\[ a_f(\infty) \propto \int E(\omega) E(\omega_{fg} - \omega) d\omega \]

Many combinations of the frequency pairs determine the total excitation

Two-photon excitation process

\[ a_f(\infty) \propto \int d\delta \omega E(\omega_0 + \delta \omega)E(\omega_0 - \delta \omega) = \int d\delta \omega |E(\omega_0 + \delta \omega)||E(\omega_0 - \delta \omega)| \cdot e^{i[\Phi(\omega_0+\delta \omega)+\Phi(\omega_0-\delta \omega)]} \]

Transition probability is controlled by the spectral phase of the incident field

At \( \omega_0 \): antisymmetric phase is unaffected by transition probability

Transform limited pulses are the most efficient:

\( \omega_0 = \omega_{fg}/2 \)

Phase coherent control of 2P processes in molecular systems

\[
TPF \propto \int \sigma^{(2)}(\omega) \left| \int E\left(\frac{\omega}{2} - \Delta\right)E\left(\frac{\omega}{2} + \Delta\right) d\Delta \right|^2 d\omega
\]

TPA large spectrum 2-photon excitation spectrum

- **Flat phase**: Optimum over the whole spectrum
- **Phase antisymmetric point**: no destructive interference
- **Elsewhere**: phase is not optimal: weak or zero-signal

Matching the molecular absorption profile

\[ \text{TPF} \propto \int \sigma^{(2)}(\omega) \left| \int E\left(\frac{\omega}{2} - \Delta\right) E\left(\frac{\omega}{2} + \Delta\right) d\Delta \right|^2 d\omega \]
Coherent control for selective two-photon fluorescence microscopy of live organisms

J.P. Ogilvie, …. E. Beaurepaire, M. Joffre, OE (2006)
Coherent control for selective two-photon fluorescence microscopy of live organisms

J.P. Ogilvie, .... E. Beaurepaire, M. Joffre, OE (2006)

Also Marcos Dantus group, Michigan State
Linear combinations yield two selective images of Drosophila embryo

Blue pulse

Red pulse

Yolk emission

GFP emission

J.P. Ogilvie, …. E. Beaurepaire, M. Joffre, OE (2006)
Phase coherent control for specific imaging: pH selectivity
Nonlinear processes involve intra-pulse interferences

\[ P^{(1)}(k, \omega) = \chi^{(1)}(\omega) : E(k, \omega) \]
\[ P^{(2)}(k = k_i + k_j, \omega = \omega_i + \omega_j) = \chi^{(2)}(\omega_i + \omega_j) : E(k_i, \omega_i) E(k_j, \omega_j) \]
\[ P^{(3)}(k = k_i + k_j + k_l, \omega = \omega_i + \omega_j + \omega_l) = \chi^{(3)}(\omega_i + \omega_j + \omega_l) : E(k_i, \omega_i) E(k_j, \omega_j) E(k_l, \omega_l) \]

SHG:
Polarized NLO imaging
Imaging bio-molecular organization

Zeiss- Human Osteosarcoma - Actin

Collagen (SHG) / elastin (TPF) chordae,
U. Exeter/I. Fresnel

Detergent extracted actin cytoskeleton SEM- Ian Wells, DB Stolz

Collagen (SEM)
Elastin (SEM)
Molecular orientational order reports structural information

Resolution in nonlinear optical microscopy: ~ 300nm

Ordered

Disorder due to Spatial rearrangements / Dynamics

ψ = 180°

Disordered
1 photon fluorescence
Fluorescence = Absorption x Emission

Linear excitation

Absorption

\[ \mu_{abs} \sim < \psi_0(r) \cdot r \cdot \psi_1(r) > \]

\[ P_{abs} \propto |\mu_{abs} \cdot E|^2 \]

\[ P_{abs} = \sigma_{abs} \cdot I \]
1PF: tuning the excitation - single molecule

\[ I(\alpha) \propto |\vec{\mu}_{\text{abs}} \cdot \vec{E}|^2 \]

Absorption dipole \hspace{1cm} exciting field

Many molecules

\[ I^{1PF}(\alpha) = N \int_{\Omega} |\mu_{\text{abs}}(\Omega) \cdot \vec{E}|^2 f(\Omega) d\Omega \]

Time and space averaging

S. Brasselet et al. in Springer Series Fluoresc, Springer-Verlag (2013)
Polarimetric Fluorescence (1PF) imaging

Objective lens
NA=1.2
Galvos
Dichroic mirror
Confocal pinhole
APD
Motorized HWP
CW Laser

\[ I(\alpha) = A_0 \left[ 1 + \frac{A_2}{A_0} \cos(2\alpha) + \frac{B_2}{A_0} \sin(2\alpha) \right] \]

\[ (A,B) \leftrightarrow (\rho,\psi) \]

Gasecka et al. Biophys J 2009
Duboisset et al. JPC B 2013
Kress et al., Biophys J 2013
Structural imaging of actin stress fibers
Alexa 488 – phalloidin in fixed COS 7 cells

\[ \Psi = 119 \pm 3 \text{ deg} \]
Structural imaging of actin stress fibers

Alexa 488 – phalloidin in fixed COS 7 cells
Structural imaging of actin stress fibers

Alexa 488 – phalloidin in fixed COS 7 cells
Structural imaging of actin stress fibers

Alexa 488 – phalloidin in fixed COS 7 cells

2 μm
Label-free
Polarization resolved Nonlinear microscopy

\[
(E_X^\omega, E_Y^\omega, E_Z^\omega) = E_0 (\cos \alpha, \sin \alpha, 0)
\]

\[
\vec{P} = \varepsilon_0 \chi^{(1)} \vec{E} + \varepsilon_0 \chi^{(2)} \vec{E} \vec{E} + \varepsilon_0 \chi^{(3)} \vec{E} \vec{E} \vec{E} + \ldots
\]

- SHG
- THG
- FWM / CARS

All dipole radiations will add up coherently
Polarization resolved nonlinear microscopy

Forward detector

2PF
FWM/CARS
SHG

sample

filters

Rotating polarization

BS

Epi detector

APD

counts

Experimental

Fit

S. Brasselet, AOP 2011
J. Duboisset et al., PRA 2012
F.Z. Bioud et al., PRA 2014
Polarization resolved SHG/TPF

\[(E^\omega_X, E^\omega_Y, E^\omega_Z) = E_0 (\cos \alpha, \sin \alpha, 0)\]

\[P_{\text{SHG}} \propto N \int [\beta(\Omega) : E(\alpha)E(\alpha)] f(\Omega)d\Omega\]

\[P_{\text{SHG}} \propto \chi^{(2)} : E(\alpha)E(\alpha)\]

\[I = a_0 + a_2 \cos (2\alpha) + a_4 \cos (4\alpha)\]

\[b_2 \sin (2\alpha) + b_4 \sin (4\alpha)\]

\[f(\Omega) = \text{circle} + \text{other terms} + ...\]
SHG polarized microscopy in collagen

60 µm

High disorder

High order

SHG Intensity

10 µm

S2, φ2
Fast SHG polarized microscopy in collagen

Sison et al. In prep (2021)
pSHG provides sub-diffraction information
Gold nanorods

\[
I = a_0 + a_2 \cos (2\alpha) + a_4 \cos (4\alpha) + b_2 \sin (2\alpha) + b_4 \sin (4\alpha)
\]

\[
I_n = \frac{1}{a_0} \sqrt{a_n^2 + b_n^2} \quad \varphi_n = \frac{1}{n} \arctan \left( \frac{b_n}{a_n} \right)
\]

Balla et al. ACS Photonics (2017)
pSHG provides sub-diffraction information
Gold nano-stars

Collaboration R. Quidant, ICFO

Balla et al. ACS Photonics (2017)
Polarization resolved CARS

$$\begin{align*}
(E^\omega_X, E^\omega_Y, E^\omega_Z) &= E_0 (\cos \alpha, \sin \alpha, 0) \\
I^\text{CARS}_X &\propto \sum_{JKL} \chi^*_{JXK}\chi^*_{XMNO} E^*_J E_K E_L E_M E^*_N E^*_O
\end{align*}$$

$$I = a_0 + a_2 \cos (2\alpha) + a_4 \cos (4\alpha) + a_6 \cos (6\alpha) + b_2 \sin (2\alpha) + b_4 \sin (4\alpha) + b_6 \sin (6\alpha)$$

$$f(\Omega) = \bigcirc + \bigcirc + \ldots$$
High orders of the molecular angular distribution

\[ \chi_{IJKL}^{(3)} = N \gamma \int_\Omega (I \cdot e)(J \cdot e)(K \cdot e)(L \cdot e) f(\Omega) d\Omega \]

2D incident polarization

\[ p(\varphi) = p_0 + \sum_{n} p_n \cos(n\varphi) + q_n \sin(n\varphi) \]

symmetry orders

\[ I = a_0 + a_2 \cos(2\alpha) + a_4 \cos(4\alpha) + a_6 \cos(6\alpha) + b_2 \sin(2\alpha) + b_4 \sin(4\alpha) + b_6 \sin(6\alpha) \]

FZ Bioud et al. PRA 2014
J. Duboisset et al. JPCB 2015
pCARS in MLVs (CH$_2$ stretch. bonds)

F.Z. Bioud et al., PRA 2014
M. Hofer et al. Optica (2017)
pCARS provides sub-diffraction information

100nm pixel size, PSF ~ 300nm

CARS EAE Score 2.5

S₂, φ₂

EM
demyelination

Fu et al. J Neurosci Res. 2007

P. Gasecka et al., Biophys. J. (2017)
NLO imaging in depth in tissues
Optical imaging at large depths

Biological tissues: $L_s \sim 100 \, \mu m$, $L_t \sim 1 \, mm$

- $z < L_s$
- $L_s < z < L_t$
- $z > L_t$

**Ballistic regime**

**Diffusive regime**

**Aberrations**

**Scattering**
Light propagation in biological media

Transport mean free path:

\[ L_t = \frac{L_S}{1-g} \]

Anisotropy:

\[ g = \langle \cos \theta \rangle \]

Biological media are forward-scattering: \( g \sim 0.9 \)
Optics in complex media

Aberrations (ballistic light)  Multiple scattering (diffuse light)

Wavefront distortions come from:

- Aberrations of the optics/sample
- Scattering
Adaptive optics

Deformable mirror and feedback loop

Image of the retina. Dr. Stephen A. Burns. Scale bar: 50 μm.
Adaptive optics and 2PF imaging

Combining fluorescence microscopy with adaptive optics in aberrant/scattering media

E. Betzig lab, Nat Meth 2014

living zebrafish brain
Imaging above Lt ? scattering media

Spatial distortions

A : illumination area

\[ N_{\text{modes}} = \frac{A}{\lambda^2} \]

Y. Silberberg
Optimization iterative process on N SLM pixels
CW Wavefront Shaping by transmission matrix inversion (TM)

Popoff and Gigan, PRL 2010

\[ E_{\text{out}}^m = \sum_{n=1}^{N} |t_{mn}| e^{i\theta_{mn}} E_n^{\text{in}} \]

CCD pixels (measured by phase step interferometry)

SLM pixels

Refocussing through a scattering medium:

\[ \tilde{E}^{\text{in}} = T^\dagger \tilde{E}^{\text{target}} \]

Laser 532nm

CCD

Controlled Part

Reference Part

Sample

Observation Window

P

P

P

D

SLM
Ultrashort pulses in a scattering medium

Spatial + temporal distortions

Spatio-temporal speckle
(Multi-path problem)

Y. Silberberg
CW vs. short pulse propagation in scattering media

Y. Silberberg
Short pulse propagation in scattering media

Medium spectral width $\delta \lambda_m$

Light confinement time $\tau_m$

(ZnO scattering thick sample)


Averaged 1000 speckle grains:

Number of spectral or temporal speckle grains (or modes):

$$N_\lambda = \frac{\delta \lambda_L}{\delta \lambda_m} = \frac{\tau_m}{\delta \tau_L}$$

M. Mounaix PhD (2016)
Spatiotemporal focusing of an ultrafast pulse by local pulse characterization through a scattering medium

The speckle is imaged on a 2D spectrometer

After phase compensation at a precise location

Space and time are TL

Spatiotemporal focusing by a spatial control

Optimization of a 2PF signal naturally optimizes spatiotemporal focusing

Through 500μm thick bone:

Initial excitation

Optimized

Spatiotemporal focusing by a spatial control

Temporal control is possible by spatial modulation

Coherent spectral control of the output pulse: Multi–Spectral Transmission Matrix

TM measured for 21 wavelengths within a 13nm spectral window around 800nm

\[ E_{j\text{out}} = \sum_{m=1}^{N_{\text{SLM}}} \sum_{l=1}^{N_{\omega}} |h_{jml}| e^{i\varphi_{jml}} E_{m}^{\text{in}}(\lambda_l) \]

Both time and space can be controlled

\[ N_{\lambda} \times N_{\text{SLM}} \]

measured spatio-spectral components of the TM

MSTM refocussing:

M. Mounaix, S. Gigan, PRL 2016
Conclusion

Efficient nonlinear microscopy imaging requires time, space and polarization control

Fundamental biological studies: there is room for optimal optical schemes for in-depth real time imaging

Clinical applications: from nonlinear microscopy to endoscopy
Thanks

Collaborators

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