Multimodal nonlinear microscopy of tissues

Two-photon (2PF) microscopy

Coherent microscopes: SHG, THG

Mulltiphoton/multicolor imaging

Imaging depth

Imaging speed

Epidetection of coherent signals

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Scanning nonlinear microscopy

Pulsed NIR beam is focused inside the sample.

Focused spot is scanned in 2D or 3D with mobile mirrors to record an image.

Animation: T. Savy

Nonlinear (=multiphoton) microscopy

Nonlinear microscopy in biology: some fields of application

Neuroscience

THG: Lipid/water interfaces, etc

THG: Organized fibers: collagen, muscle, etc

Different multiphoton processes

Excitation: focused pulsed infrared light

Extraction: 1 photon excited fluorescence

2PEF: 2 photon excited fluorescence

Advantage for tissue imaging

2PEF microscopy: Dark & Webb (Cornell Univ. 1990)

1-photon excited fluorescence

Excitation: focused pulsed infrared light

(700-1300nm, 100fs, 1-100MHz)

2-photon excited fluorescence

Deep imaging

(Max 0.4 - 0.6mm)

[+] keeps 3D micron-scale resolution.

[+] confined photoperturbation

2PEF microscopy: Denk & Webb (Cornell Univ. 1990)

Pulsed lasers are used for optimal multiphoton excitation with minimal average power

Pulses are typically ~100 femtoseconds FWHM

T: Ti:Sapphire laser

Deep imaging

Gain with pulsed excitation:

T: Ti:Sapphire laser

E = FWHM

FWHM = 10 ns

≈ 10 ns

Gain with pulsed excitation:

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THG, 3HG, and SHG imaging of the human cornea.

**3HG (3rd harmonic) contrast: heterogeneity**

- **Homogeneous medium**
  - no 3HG!
  - interference partially constructive
- **Interface between different media**
  - 3HG detects interfaces!

Visualization of tissue morphology (without labeling)

- Contrast: interfaces between cells and extracellular medium:
  - Lipid membranes, ...

Detection of interfaces (3HG) → 3D morphology

- Visibly cell contours
- Calculation of 3HG as a function of size

**SHG probes the macromolecular organization of collagen**

- Molecules
- Macromol. organization
- Tissue

  - Fibrous matrix (Collagen I)
  - Non fibrous matrix (Collagen IV, ...)
  - No SHG

- SHG sensitive to laser polarization and molecular orientation

- Source of signal
  - Molecular origin: peptide bonds
  - First level of order: organization of peptide bonds in a helical pattern
  - SHG, 2PEF: two-photon-excited fluorescence

- More info → nonlinear imaging with broadband excitation

- Anti Col - FITC
- Anti Col IV - FITC
- SHG probes the macromolecular organization of collagen

**Example: multimodal imaging of human cornea**

- Z-stack 2PEF, THG, SHG
- Imaging cell divisions in the zebrafish embryo

- SHG provides landmark with sub-minute precision

- More contrast mechanisms → additional information

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Visibility of cell contours

Calculation of 3HG as a function of size

**Imaging cell divisions in the zebrafish embryo**

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**2PEF, two-photon processes**

- Planar excitation
- Excitation spectrum

- THG, 3HG: second-harmonic generation sensitive to interfaces and heterogeneity

- CARS, SRS: «nonlinear Raman» molecular vibration modes (eg CH2 stretch)

**Strategy 1: two-color wave mixing with combined pulses**

- 2PEF, two-photon processes
- SHG (2HG): second-harmonic generation sensitive to asymmetry at sub-µm scale
- THG (3HG): third-harmonic generation sensitive to interfaces and heterogeneity

**Strategy 2: shaped broadband pulses**

- Pump-probe microscopy
  - excited-state absorption (eg melanin, Hb)

More contrast mechanisms → additional information
**Multicolor Multiphoton Microscopy for Volume and Live Imaging**

Some situations requiring multiple λ:
- Image several FPs: GFP+mCherry+… labeling
- Photoactivation
- Quantitative FRET
- Brainbow: genetic engineering strategies for tracing cell lineage and neuronal circuits

**Non Resonant (nm)**

**Multiphoton Microscopy** for volume live and imaging

Mixed wavelengths to address mixed xFPs

- Brainbow: genetic engineering for tracing cell lineage and neuronal circuits
- Some situations requiring multiple λ:
  - Image several FPs: GFP+mCherry+… labeling
- Photoactivation
- Quantitative FRET

**Brainbow**:
- Genetic engineering for tracing cell lineage and neuronal circuits
- Some options:
  - Wavelength tuning
  - 3 (or more) femtosecond lasers
  - Excite several dyes with single λ
- Increased bleaching
- Wavelength mixing

**CARS is not background free**

**CARS** involves using two laser frequencies to interact with a specific molecular vibration

CARS signals are generated at wavelengths shorter than the excitation wavelength (anti-Stokes)

**Implementation example: two picosecond pulse trains (oscillator + OPO)**

**Nonlinear imaging with broadband excitation**

Strategy 2: Shaped broadband pulses

**Label-Free Biomedical Imaging with High Sensitivity by Stimulated Raman Scattering Microscopy**

Advantages of SRS over CARS:
- SRS is background free
- Linear dependence on concentration (better for small concentrations)
- Directly gives Raman spectrum
- BUT more complex
  - Note: A CARS setup can be converted to SRS
  - CARS still good for lipid studies

10 MHz modulation and lock-in

**What limits the imaging depth?**

Confined excitation even in scattering media … but the number of ballistic excitation photons decreases exponentially with depth

| Signal: |
| \[ S = \frac{T}{t} \left( \frac{P_0}{P_{in}} \right) \Phi(z_{max}) T/\tau \] |
| **average laser power** |
| \( z_{max} = \left( \frac{L_{ex}}{\alpha} \right) \ln(\alpha P\sqrt{\Phi(z_{max}) T/\tau}) \) |
| \( \alpha \)- fluorophore efficiency and detector noise |
| \( \Phi(z_{max}) \)- generated fluorescence typically \( \approx 600 \mu m \) (layer 2/3 neocortex) |
| \( T \)- inverse laser cycle duty |

Endogenous GFP RFP THG

100 µm

**Live Multicolor 2P Imaging: Chick Embryo Spinal Cord**

Developing spinal cord tissue slice

CARS process (vibration-specific)

- Resonant (signal)
- Non resonant (non-specific)

FWM (non-specific)

CARS intensity:

\[ I_{CARS} \approx \Delta \Phi \left( \frac{P_{in}}{P_{0}} \right) \Phi(z_{max}) T/\tau \]

One way to circumvent this difficulty -> SRS microscopy

**CARS** is mixed with these other non-specific wave-mixing processes

Polarization:
- Resonant (signal)
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Implementation example: two picosecond pulse trains (oscillator + OPO)

**Excitation**

- 1100 900 1000

**CFP-YFP-tdTomato**

Excitation

- Photoactivation

Mahou et al, Nat Methods 2012

Some options:
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Increased bleaching
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Deep tissue multiphoton microscopy using longer wavelength excitation


“Transparence window” of tissues

Near-IR excitation limits absorption and scattering:

- Reduced perturbation of samples
- Good penetration in tissues (IR excitation, λ = 0.7-1.3 µm) (scattering mean free path ~100-200 µm)

Multiphoton microscopy:

- At large depths: contrast & resolution loss
- Good penetration in tissues (IR excitation, λ = 0.7-1.3 µm)

How to increase imaging depth?

\[ z_{\text{max}} = L_{\text{max}}^{(c)} \ln(\alpha \bar{P} \sqrt{\phi(z_{\text{max}})} T / \tau) \]

- 3. Increase collection efficiency (\( \phi \))?

Typical multiphoton microscope

- Non-descanned > detection (close to objective)

Increasing penetration depth: conclusion

Excitation
- Longer excitation wavelength
- Tissue clearing
- Amplified pulses
- Wavefront control (adaptive optics)

Collection
- Low magnification, high NA to improve collection

(However: Low mag. objective multiplies by 10 -> equivalent to multiplying P by “only” 3)

→ Reaching fundamental limits for 2PEF beyond 1mm

Loss in contrast and resolution
**Multiphoton microscopy in practice**

- **A few messages**
  - Near-field microscopy provides 3D tissue imaging with 0.5-2-4µm resolution
  - Imaging depth: a few 100µms. Up to 1mm?
  - SHG obtained from fibrillar collagen and myofilaments; probes macromolecular organization; THG provides images of tissue morphology
  - Femtosecond laser-based methods are "easily" combined: multicolor 2PEF, mHG, laser ablation, etc

**A few references**

"Nonlinear magic: multiphoton microscopy in the biosciences"  

"Advances in multiphoton microscopy for imaging embryos",  

"Deep tissue two-photon microscopy"  

"SH imaging microscopy for visualizing biomolecular arrays in cells, tissues and organisms"  

**More refs → www.lob.polytechnique.fr**

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**Faster → parallelisation**

<table>
<thead>
<tr>
<th>2p-microscopy</th>
<th>2PEF (standard)</th>
<th>Resonant scanning</th>
<th>Multifocal Light sheet (SPIM)</th>
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<tbody>
<tr>
<td>Pixel rate (speed)</td>
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<td>Accumulation time per voxel</td>
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<td>Laser average power</td>
<td>P</td>
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**High-content imaging with 2P-SPIM**

**A few messages**

- Check NIR transmission of microscope.
- Use adapted objective (transmission, working distance, etc)
- Use optimal excitation wavelength (GFP: 940-960nm)
- Detection filters # confocal microscopy: with a single fluorophore, short pass filter (ie SP80) to optimize collection
- Non-descanned detection / no need for pinhole
- PMT close to objective
- Epi-detection for thick sample, epi+trans otherwise.
- In short: do not use the same settings for confocal and multiphon microscopy!