MULTI-PHOTON MICROSCOPY: APPLICATIONS AND THEORY

PART II

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FLUOVIEW LASER SCANNING CONFOCAL AND MULTIPHOTON
SINGLE PHOTON VERSUS TWO PHOTON IMAGING
TWO-PHOTON DISCOVERY

MARIA GOEPPERT-MAYER FIRST DESCRIBED THE PROCESS OF TWO-PHOTON ABSORPTION IN HER DISSERTATION IN 1931 (UNIVERSITY OF GÖTTINGEN). MATHEMATICALLY PREDICTED. 2P LASERS WERE NOT INVENTED UNTIL 1960’S. LATER RECEIVED NOBEL PRIZE FOR WORK ON ATOMIC NUCLEAR SHELL THEORY.

2-P MICROSCOPY PIONEERED BY WINFRIED DENK IN THE LABORATORY OF WATT WEBB AT CORNELL.

2-P PATENT WAS HELD BY DENK, STRICKLER, WEBB 1991 (RECENTLY EXPIRED).
GM UNITS FOR 2P-CROSS SECTIONS
NUMERICAL APERTURE

\[ \text{NA} = n \sin \mu \]

Fluorescence Intensity \( \approx \frac{\text{NA}^4}{\text{Mag}^2} \)

Super 25x Water Objective MPE
\[ \text{NA} = 1.05 \quad \text{Mag} = 25x \]

UPLANFL 20X Dry Objective
\[ \text{NA} = 0.5 \quad \text{Mag} = 20x \]

Fluorescence Intensity \( \approx 12.4x \)
Resolution \( \approx 2.1x \)
\[ (R = \frac{\lambda}{2\text{NA}}) \]

 refractive index
Air = 1.0
Water = 1.3
Oil = 1.5
Airy Patterns and the Limit of Resolution

- Resolution Limit
- Unresolved
- Resolved
- Airy Patterns
- Airy Disks

3-Dimensional Point Spread Function
# Emission Wavelength and Resolution

<table>
<thead>
<tr>
<th>Wavelength (Nanometers)</th>
<th>Resolution (Micrometers)</th>
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<tbody>
<tr>
<td>360</td>
<td>.19</td>
</tr>
<tr>
<td>400</td>
<td>.21</td>
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<tr>
<td>450</td>
<td>.24</td>
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<td>500</td>
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<td>550</td>
<td>.29</td>
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<td>600</td>
<td>.32</td>
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<tr>
<td>650</td>
<td>.34</td>
</tr>
<tr>
<td>700</td>
<td>.37</td>
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FV1000MPE components

- Confocal detectors
- Pulsed TiS laser
- AOM
- Motorized beam expander
- Reflected detector
- Forward detector
SINGLE PHOTON (CONFOCAL) VS. MULTI-PHOTON EXCITATION
Lasers for MPE microscopy

- Almost all lasers used for MPE microscopy are Titanium sapphire or Ti:sapphire lasers.

- Typical pulse durations range from 80 femtoseconds (fsec) to 140 fsec.

- Typical bandwidth is from 690 to 1040nm, limited by the falloff in laser power available at the extremes of the range and other factors.

- At a typical repetition frequency of 80MHz, there are 134 pulses per pixel at 1 frame/sec at 512 x 512 pixels resolution.
Image Collection In Confocal vs MP

Confocal Microscope

Multi-Photon Microscope

No need for confocal aperture

Excitation only at the focal plane
Simple optical path and optimized optics for efficient 2P detection.
RELATIONSHIP BETWEEN IMAGE INTENSITY AND LASER POWER AND PULSE WIDTH

\[ I \propto \frac{P^2_{ave}}{\tau_{sample}} \]
Dispersion and Pulse Duration

- **Pulse Duration After Dispersion**
- **Original Pulse Duration**

\[ \tau_{\text{sample}} = \tau_0 \sqrt{1 + \left( \frac{4 \ln 2 \cdot \text{GVD}}{\tau_0^2} \right)^2} \]

DeepSee® – Short Pulses Delivered to Sample

- Mai Tai HP Pulse duration

![Graph showing pulse duration and wavelength relationship.](image-url)
RELATIONSHIP BETWEEN IMAGE INTENSITY AND LASER POWER AND PULSE WIDTH
RELATIONSHIP BETWEEN IMAGE INTENSITY AND LASER POWER AND PULSE WIDTH

Same power on sample (~1.5 mW @ 830 nm), same PMT settings

Without Dispersion Compensation

With Dispersion Compensation

Red: RFP stained tumor cells (575-640 nm), Green: FITC-dextran inj. vessels (500-550 nm)
Excitation wavelength: 830 nm, AOM plus 40× / 0.8W objective
LINEAR AND NONLINEAR IMAGING MODALITIES

1PEF: 1-Photon Excited Fluorescence
2PEF: 2-Photon Excited Fluorescence
3PEF: 3-Photon Excited Fluorescence
SHG: Second Harmonic Generation
THG: Third Harmonic Generation
CARS: Coherent Anti-Stokes Raman Scattering

1PEF ~ I
2PEF & SHG ~ I^2
3PEF & THG ~ I^3
CARS ~ I_p I_s

http://www.its.caltech.edu/~supatto/research02.htm
SECOND HARMONIC GENERATION (SHG)

HIGHLY ORDERED MOLECULAR STRUCTURE

NONCENTROSYMMETRIC MOLECULAR STRUCTURE
- NO INVERSION CENTER

LIGHT EXITING SHG MATERIAL IS:
½ \( \lambda \) AND 2E OF LIGHT ENTERING SHG MATERIAL

860nm excitation -> 430nm emission at 2x ENERGY

MUSCLE, COLLAGEN, POLYSACCHARIDES

ENERGY CONSERVED - MOLECULES ARE NOT EXCITED

NO PHOTOBLEACHING AND NO PHOTOTOXICITY

LABEL FREE
Second Harmonic Generation

Collagen Emission with 800nm TPE
SECOND HARMONIC GENERATION

Linear

Nonlinear (or multiphoton)

1PEF: 1-Photon Excited Fluorescence
2PEF: 2-Photon Excited Fluorescence
3PEF: 3-Photon Excited Fluorescence
SHG: Second Harmonic Generation
THG: Third Harmonic Generation
CARS: Coherent Anti-Stokes Raman Scattering
SECOND HARMONIC GENERATION IMAGING

MIP xyz
25X 860nm excitation  (LABEL FREE)
COLLAGEN INSIDE OF INTACT MOUSE LYMPHNODE
RELATIONSHIP BETWEEN IMAGE INTENSITY (SHG) AND PULSE WIDTH

62 fs

90 fs

170 fs

140 microns deep
SECOND HARMONIC GENERATION

Polysaccharide (starch) packets in a slice of raw potato

25x

MIP xyz

LABEL FREE

860nm
C. elegans Second Harmonic Generation Imaging

Epi-SHG

Forward-SHG
SECOND HARMONIC GENERATION

MOUSE MUSCLE
SHG
25X

LABEL FREE
INTRAVITAL
860nm EXCITATION
SHG AND TPEF/2PEF

1PEF: 1-Photon Excited Fluorescence
2PEF: 2-Photon Excited Fluorescence
3PEF: 3-Photon Excited Fluorescence
SHG: Second Harmonic Generation
THG: Third Harmonic Generation
CARS: Coherent Anti-Stokes Raman Scattering
Second Harmonic Generation and TPEF

860nm excitation

Skin graft infected with GFP encoding virus

SHG

Emission in violet and green

25x

DEEP
Rat skin

800nm

E-SHG
TRITC
AUTO
AUTO

25x
Second Harmonic Generation and TPEF-AUTOFLUORESCENCE

800nm excitation
Emission in violet and green
MIP XYZ
Human skin section
25x
Label free
Second Harmonic Generation and TPEF-AUTOFLUORESCENCE

800nm excitation
Emission in violet and green

C. elegans
25x
Label free
Alzheimer’s Plaques
Amyloid in red
SHG in grey
DAPI in white/grey

Stitching upgrade
Transgenic zebrafish expressing GFP in pancreatic beta cells.

GFP
Anti-insulin
E-SHG
F-SHG

Dr. Stef Nalle
Dr. Vicky Prince
Membrane Potential using FM4-64 SHG

MPE 950nm → DM505 → Fluorescence at 605-680nm

SHG at 475nm

Relative Change in SHG (%)

Voltage (mV)
COMBINING IMAGING OF MEMBRANE POTENTIAL (SHG) WITH IMAGING pH (2P FLUORESCENCE)
2PEF/TPEF

- Linear:
  - 1PEF: 1-Photon Excited Fluorescence
  - 2PEF: 2-Photon Excited Fluorescence

- Nonlinear (or multiphoton):Order 2
  - 2PEF & SHG: $I^2$
  - 3PEF & THG: $I^3$
  - CARS: $I_p^2I_s$

- Order 3
  - 3PEF: 3-Photon Excited Fluorescence
  - THG: Third Harmonic Generation
  - CARS: Coherent Anti-Stokes Raman Scattering

SHG: Second Harmonic Generation
GFP-LABELED GLOMERULUS
DEXTRAN-TEXASRED VESSELS

INTACT KIDNEY FROM A TRANSGENIC MOUSE
FOLLOWING CARDIAC PERFUSION WITH DEXTRAN-TEXASRED
Drosophila testes

T 0ms
Transgenic GFP-IP3R3 in olfactory bulb
Human intestine
GFP Neuron Spines
INTRAVITAL GFP TUMOR CELLS 2PEF

GFP LABELED TUMOR CELLS INJECTED INTO MOUSE

960nm  Z-series  Optical sectioning  MIP XYZ
ZEBAFISH HEAD

920nm GFP 2PEF
LABELED ZEBRAFISH HEAD
RACHEL WONG LABORATORY
INTRAVITAL IMAGING CONSIDERATIONS

MOVEMENT
HEART BEAT
BREATHING
RUNNING

ANIMAL SURVIVAL
BODY WINDOWS
VENTILATOR
ANESTHESIA

UPRIGHT VS. INVERTED

DEPTH OF IMAGING

TTL TRIGGERING
OF ACQUISITION

TIME RESOLUTION
MICROPROBE OBJECTIVES

- wide-angle 6X magnification (NA 0.14)
  1.3 mm-diameter lens

- high-resolution 20X magnification (NA 0.5)
  1.3 mm-diameter lens

- high resolution 27X magnification (NA 0.7)
  3.5 mm-diameter
INTRAVITAL IMAGING

DEXTRAN-TEXAS RED LABELED BLOOD VESSELS
TUMOR CELLS LABELED WITH GFP

VISUALIZING METASTASIS

25X LIVE MOUSE UNDER ANESTHESIA
920nm excitation
LABEL FREE LIPID IMAGING USING CARS (UPGRADE FOR FV1000MPE)

COHERENT ANTI-STOKES RAMAN SCATTERING (CARS) MICROSCOPY

IMAGING LIPIDS (CH2) WITHOUT STAINING OR FLUORESCENT TAGS

COMBINE WITH OTHER (LABEL FREE) TECHNIQUES

INTRINSIC FLUORESCENCE
FLUORESCENT PROTEIN OR DYE
SHG
COHERENT ANTI-STOKES RAMAN SCATTERING (CARS) PRINCIPLE

- Two excitation wavelength, $\omega_p$ (pump) and $\omega_s$ (Stokes). The wavelength difference ($\Omega$) matches vibration frequency.
- It is “coherent”. The signal appears in a specific direction (forward or backward)
  - The first two light-matter interaction prepared the coherent state. The third interaction create Raman scattering.
- NIR excitation, high optical resolution
Coherent Anti-Stokes Raman Spectroscopy (CARS)
PROPERTIES OF CARS SIGNAL

• CARS signal can be detected in Epi and Forward direction.
  ‣ Called E-CARS and F-CARS signal.
  ‣ E-CARS signal is weak relative to FCARS, but sensitive to small features
  ‣ F-CARS is strong due to coherent addition
RAMAN SPECTROSCOPY INSIDE A CELL

Strong CH₂ signal
GENERATING THE PUMP AND STOKES BEAMS WITH A PCF
LOW DISPERSION WITH THE CARS PCF
Simultaneous Forward CARS/Second Harmonic Generation Detection

Excitation: Pump 800nm
Stokes 1040nm

PMT

SHG: 350-430nm

DM 485nm

CARS: 637-663nm

Emission
Multimodal Imaging using FV1000MPE

Porcine muscle tissue with no staining

RED : CARS (lipid)
GREEN : TPE
WHITE : SHG (collagen)
CARS imaging of lipids in vivo

Myelin sheath (70% lipid) of rat dorsal root nerve

Image courtesy of: Dr. Albert Stolow (NRC, Canada)
High-fat fed mouse fat imaged with F-CARS, E-CARS, and TPEF.

Maximum Intensity Projections.

800nm Pump
1040nm Stokes

Super 25X 1.05NA

Scale Bar = 100µm

Olympus FV1000MPE-CARS
CARS AND SHG

Porcine
Super 25x
Epi-CARS
SHG
CARS AND SHG

Porcine
Super 25x
Epi-CARS
SHG
CARS AND SHG

Salmonoides
Super 25x
Epi-CARS
Second Harmonic Generation
Early cancer metasis labeled with GFP (GREEN) invading liver tissue visualized with Forward-CARS (PURPLE) and second harmonic generation (BLUE) in a nude mouse.
Breast cancer tissue

800nm pump
1040 stokes

E-SHG
F-SHG
CARS

25x

Scale bar = 100um
Intestinal villi from olive oil gavaged mouse.

E-CARS
Autofluorescence
Label free
25x
MIP
CARS Lipid Imaging Applications

Lipids are hard to image

- Extracted during fixation
- Extrinsic dyes can disrupt structure
- No antibodies

Biological Relevance, areas of Study

- lipid-droplet biology
- lipid metabolism in simple organisms
- obesity-cancer-lipids in metastasis relationship
- atherosclerotic lesions
- lipid-rich structures including myelin sheath, skin, and brain
Combining Electrophysiology with Imaging

(a) Image of a neuron with a marker indicating Uncage and Alexa594.
(b) Time series of fluorescence intensity with markers H, D, and Uncage.
(c) Image of OGB-5N with markers H, D.
(d) Image of calcium concentration change with markers H, D.
(e) Graph showing calcium concentration change over time with markers H, D, and Uncage.
Combining Electrophysiology with Imaging
NEW OLYMPUS 25X MPE OBJECTIVE

Super 20x Dipping Objective
0.95NA 2mm WD

Super 25x MP Dipping Objective
1.05NA 2mm WD
Scale: a chemical approach for fluorescence imaging and reconstruction of transparent mouse brain

Hiroshi Hama¹, Hiroshi Kurokawa¹,², Hiroyuki Kawano¹,³, Ryoko Ando¹, Tomomi Shimogori¹, Hisayori Noda¹,⁴, Kiyoko Fukami², Asako Sakaue-Sawano¹,³ & Atsushi Miyawaki¹,³
OLYMPUS 25x MPE Objective

NA 1.05 Water WD 2.0
OLYMPUS Scaleview 25x MPE Objective

NEW!
Olympus
Scaleview 25x MPE Objective
NA 1.0 Scale-A2 WD 4.0

Pial surface

Cerebral cortex

4.0 mm (3.2 mm)

White matter

CA1

DG

Hippocampus
Olympus
Scaleview 25x MPE Objective
NA 1.0 Scale-A2 WD 4.0
NEW Olympus Scaleview-A2 Clearing Reagent
Clearing Reagent Comparison:
Olympus Scaleview-A2 vs. BABB

C

\textit{th}y1-\textit{YFP} line H

\text{Scaleview-A2} \hspace{1cm} \text{BABB}
The deepest yet...
8mm MPE Imaging

Mag = 25x
N.A. = 0.9
W.D. = 8mm

Coming in February, the 8mm WD companion to the new SCALEVIEW 25x Objective Your Image.

SCALEVIEW-A2 Optical Clearing Agent

Mouse brain (Left) and after 2 weeks incubation in SCALEVIEW-A2 (Right).
FV1000MPE IMAGING SYSTEM OFFERS NEW CAPABILITIES

Visible Confocal -
Visible spectral confocal imaging for multi-color high resolution optical sectioning.

MPE-
Intravital imaging in upright and inverted configurations. Deep tissue imaging. Extended timelapse.

SHG-
Collagen, muscles, electrical potential, Alzheimer’s plaques.

*ALL FORWARD AND REFLECTED MPE IMAGING