Information

Information given here about 2 Photon microscopy were mainly taken from these sources:

Background information on 2-Photon microscopy:
http://micro.magnet.fsu.edu/primer/techniques/fluorescence/multiphoton/multiphotonintro.html

The microscopes:
Zeiss LSM 710 NLO; http://www.zeiss.com
Olympus Fluoview 1000 MPE, http://www.olympusamerica.com

Spectra-Physics Laser:
Schematic drawing of LSM
Why use 2-Photon microscopy?

- Multiphoton
- LSM/ widefield

Single photon vs Multi photon images at different depths (40μm, 90μm, 320μm).
A multiphoton microscope gives you the opportunity to get images from deep (e.g. 500 nm) within (living) tissue, whilst photodamaging only the imaged volume.

A Multiphoton microscope is a point scanning system which excites fluorophores within the Focus volume only. Therefore you collect emission light from this volume only, enabling you to acquire optical slices, without the use of confocal pinholes.

Beside this, one is able to photomanipulate tissue/cells within a very small volume.
THE THEORY OF 2PM
Illuminate a fluorophore with appropriate $\lambda$ of light
- 1 (excitation) photon absorbed gives 1 emission photon
- BUT
  - emission photon will have less energy i.e. longer $\lambda$ than excitation photon
  - it’s $\lambda$ and energy vary due to which $S_0$ level (0,1,2,3) the fluorophore relaxes
- Fluorescence - photons with different $\lambda$ emission curve is bell shaped
Theory for 2PM: $\lambda \sim E$ - The Energy of a Photon

\[ E = \frac{hc}{\lambda} \]
\[ E = 1.6 \times 10^{-19} \text{ J} \]

- **$h$:** Planck Constant: $6.626 \times 10^{-34} \text{ J} \cdot \text{s}$
- **$c$:** Speed of light: $299792458 \text{ m/s}$
- **$\lambda$:** Wavelength in nm
- **$eV$:** Electron Volt: $1.6 \times 10^{-19} \text{ J}$, gain of energy when an unbound electron is accelerated by an electrostatic potential difference of 1V

<table>
<thead>
<tr>
<th>eV</th>
<th>nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.39</td>
<td>100</td>
</tr>
<tr>
<td>6.20</td>
<td>200</td>
</tr>
<tr>
<td>4.13</td>
<td>300</td>
</tr>
<tr>
<td>3.09</td>
<td>400</td>
</tr>
<tr>
<td>2.47</td>
<td>500</td>
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<tr>
<td>2.06</td>
<td>600</td>
</tr>
<tr>
<td>1.77</td>
<td>700</td>
</tr>
<tr>
<td>1.54</td>
<td>800</td>
</tr>
<tr>
<td>1.37</td>
<td>900</td>
</tr>
<tr>
<td>1.23</td>
<td>1000</td>
</tr>
<tr>
<td>1.12</td>
<td>1100</td>
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<tr>
<td>1.03</td>
<td>1200</td>
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<tr>
<td>0.95</td>
<td>1300</td>
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<tr>
<td>0.88</td>
<td>1400</td>
</tr>
<tr>
<td>0.82</td>
<td>1500</td>
</tr>
<tr>
<td>0.74</td>
<td>1600</td>
</tr>
</tbody>
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1p 400nm = 2p 800nm = 3 eV
Theory for 2PM: How to excite (Tryptophan)

Single-photon
1 photon, 280 nm
4.5 eV
No laser for this...

\[ \frac{A}{B} = \frac{8\pi n \nu^3}{c^3} \]

Two-photon
2 photon, 580 nm
2.13 eV x2
4.26 eV

Three-photon
3 photon, 840 nm
1.47 eV x3
4.41 eV

virtual state
VERY short
0.01 fsec
(10^{-17} sec)

2-PM hypothesis introduced by
Maria Göppert-Mayer, doctoral thesis 1931
Theory for 2PM: $\lambda \sim E$ - The Energy of a Photon

Observe: range of overlap of potential Excitation
760nm: excite A488 & A633 *

for multicolor 2PM choose fluorophores so that they do overlap in excitation BUT NOT emission

* has to be checked on microscope

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Absorption</th>
<th>Emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor 350</td>
<td>720-800</td>
<td>440</td>
</tr>
<tr>
<td>Alexa Fluor 488</td>
<td>720-800</td>
<td>515</td>
</tr>
<tr>
<td>Alexa Fluor 546</td>
<td>720-840</td>
<td>569</td>
</tr>
<tr>
<td>Alexa Fluor 568</td>
<td>720-840</td>
<td>596</td>
</tr>
<tr>
<td>Alexa Fluor 594</td>
<td>720-850</td>
<td>610</td>
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<tr>
<td>Alexa Fluor 633</td>
<td>720-900</td>
<td>647</td>
</tr>
<tr>
<td>AMCA</td>
<td>780-800</td>
<td>444</td>
</tr>
<tr>
<td>bis-MSB</td>
<td>680-750</td>
<td>420</td>
</tr>
<tr>
<td>Bodipy</td>
<td>900-950</td>
<td>512</td>
</tr>
<tr>
<td>Calcium Crimson</td>
<td>900</td>
<td>615</td>
</tr>
<tr>
<td>Calcium green</td>
<td>780-850</td>
<td>531</td>
</tr>
<tr>
<td>Cascade Blue</td>
<td>750-800</td>
<td>420</td>
</tr>
<tr>
<td>Coumarin 307</td>
<td>780-800</td>
<td>530</td>
</tr>
<tr>
<td>CY2</td>
<td>780-800</td>
<td>506</td>
</tr>
<tr>
<td>CY3</td>
<td>780</td>
<td>565, 615</td>
</tr>
<tr>
<td>CY5</td>
<td>780-820</td>
<td>670</td>
</tr>
<tr>
<td>Dansyl Hydrazine</td>
<td>700-750</td>
<td>440</td>
</tr>
</tbody>
</table>
The 780nm NIR Laser might/will excite all three fluorophores, the Instrument has to unmix the mixture of Blue/Green/Red, or we have to use better fluorophore combination.
Reminder – simultaneous vs sequential scanning

Simultaneous Excitation
Resulted in artifact
Due to bleeding through on “green” image, where the “blue” appears and on the red image where the “green appears”

Sequential scanning
Does not show such Artifacts, therefore in THIS sample the Excitation are far apart.
Multicolor imaging in 2P

Simultaneous scan excites several fluorophore at once, emission is guided by filter and beamsplitter to PMTs. If FL-green bleed over into PMT of FL-red it will be seen here (in red). Sequential scan excites and collects one fluorophore at a time.!

Be sure that 488 does result in emission of FL red in the "green range"... Test that...

1) 488 Image "1"
2) 561 Image "2" final image
Lambda Scan with LSM – linear unmixing

Linear Unmixing determines the relative contribution from each fluorophore for every pixel of the image. And recalculates an image for Fluorophores used.
WHY USE 2P?
- to see deeper

Nikon instruments
See deeper – scattering problem

NIR light: 700-1100nm travelling through Specimen to focal plane will not scatter and disperse* as much as light of shorter $\lambda$ (350-633 nm for FL microscopy)

- excitation of fluorophores in greater depth

**Problem:** different fluorophores need its own NIR Laser?

**Solution:** Laser can be tuned from e.g. 690 to 1040 nm, fluorophores have wide excitation range in 2PM

*(due to different refractive indices of the various components in specimen)*

See also: Optical Clearing

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Blue light gets easily scattered by particles. Otherwise Sinatra c/would’nt sing "Blue skies, smilin' at me, nothin' but blue skies do I see"
See deeper – absorption problem

Tissue optical window: 700nm-900nm
(absorption of hemoglobin/tissue component and water)
See deeper

XYZ images of mouse brain sections expressing GFP, comparing single-photon 488 nm excitation and two-photon 910 nm excitation.

With single photon excitation, tissue can be observed only to a depth of about 90 μm, but with two photons, observation to a depth of about 320 μm is possible.

Items displayed in color are vertical cross sections of 3-dimensionally constructed images.

Specimens provided by:
Kimihiko Kameyama, Tomoyo Ochiishi, Kazuyuki Kiyosue, Tatsuhiko Ebihara
Molecular Neurobiology Group, Neuroscience Research Institute, National Institute of Advanced Industrial Science and Technology, Japan

Brochure, OLYMPUS, FV1000MPE
WHY USE 2P?

- small excitation volume, no pinhole

Matyas Molnar
Small focus spot

Multiphoton

LSM

Two-Photon event occurs only in focus volume

- All emission light is directly from focus

Resolution is similar (or worse) to LSM

- 0.3x1µm ellipsoid (high NA objective)

Penetration depth depending on specimen and optical parameter but might be up to nearly 1mm

These features will be important for various live cell imaging techniques, like bleaching, photodamaging, uncaging ...

That's why Multiphoton is also named Nonlinear.
Chance for 2PM event drops drastically with distance to focus
Small focus spot

Laser of LSM scans through specimen

Laser of 2PM scans through specimen

excitation/emission and photodamage/heat

- occurs within specimen
- also outside the focal plane

- occurs within specimen
- only in the focal plane
What is the chance that 2 photons hit the same fluorophore at almost the same time?

- a matter of time and area
- The probability of observing a two-photon absorption event on a bright sunny day is 1 per 10,000,000 years, whereas the one-photon absorption takes place every second

**Time** → the virtual state
→ Δt of intermediate virtual state = 10 attosec (10^{-17} s)
→ 1 attosecond (10^{-18} s) is the time window
→ light travels 3 hydrogen atoms within 1 attosec

**Area** → the fluorophore
→ quite small target

**Problem:** Light can not travel faster than speed of light

**Solution:** More photons are needed (high density of photons)

We need a million times more photons than in single photon fluorescence and ”good” objectives
Problem: 1 million times more photons? Very strong laser. There is no continuous wave laser to achieve this.

Solution: A moderate Laser with high photon intensity pulses
- low average power (0.3 - 2.5 W)
- high peak power (30-300 kW) pulses 50-100 fs wide
- pulse frequency 80 Mhz (1 pulse/12.5 ns)
This laser is dangerous when used (Class 4)!

Problem: Many fluorophores but one Laser
Solution: To excite a wide range of fluorophores the laser is tuneable for e.g. 700-1040 nm

Pulsed NIR Laser is tuneable for excitation wavelength twice the 1Photon-excitation wavelength
Principle of 2P excitation

- Objective
- Aperture of objective
- Specimen
- Focal plane of objective (depth of focus), light is focused here
Principle of 2P excitation

Laser pulse is far from focal plane, photon density is low, no chance for two photons to hit a fluorophore in one time.
Principle of 2P excitation

Laser pulse is closer to focal plane, photon density is more concentrated but still low, no chance for two photons to hit a fluorophore in one time.
Principle of 2P excitation

Laser pulse reached the focal plane, photon density is high, high probability for 2 photons to hit one fluorophore within 10 attosec
Principle of 2P excitation

The lucky ones emit fluorescence like they were hit by 1 high energy photon instead of 2 low energy photons.

Excitation / emission occurs only in Focal plane / spot.
Principle of 2P excitation

Laser pulse leaves focal plane, NO incident of two photons hitting one fluorophore
Principle of 2P excitation

Laser pulse disperses in tissue, NO incident of two photons hitting one fluorophore
Principle of 2P excitation

REMEMBER

Excitation / emission occurs only in Focal plane / spot confocal image without a pinhole
Recapitulate:

- NIR Laser to reach deep
- Excitation of "normal" fluorophores via 2P effect
- NIR is tuneable over range e.g. 690 nm – 1040 nm
  - 2P is only happening in focal volume
  - Ex/Em/photodamage only at focal volume

Applications:
Living animals
Manipulation of "precise" small volumes
Non-linear effects
Relax...

Light travels $300,000,000$ m / s
→ $7.4 \times$ around earth in one s
(40.075 km circumference)

The Femtosecond Laser:
Pulses of 100fs
$1 \text{ fs} = 1 \times 10^{-15} \text{ sec}$

But only 300 nm in one femtosecond
Or 30µm within 100 fs

circumference of human hair
53-565 µm

Multiphoton microscopy

Objectives and Detectors

Light must come in to depth
Light must get collected from the depth
Bring back home the photons

Laser → Objective → Excitation

Emission → Objective → Detector

Low NA → High NA

Objective → Detector
Multiphoton objectives

- Long Working distance (2mm) including (!)
- High Numerical Aperture
  (good resolution/focus, narrow depth of focus)

- All photons to the focus for high chance of 2P-Ex

- High transmittance and correction for broad range of e.g. 400 nm to 1000 nm
- Water dipping (remember *in vivo* imaging) / cover slip
- Correction collar (!) to compensate for different refractive indices (water 1.3, specimen 1.34-1.4)
- 34 degree angle at lense top for better accessibility to specimen for manipulation

The Olympus XLPlan N
25x, NA 1.05
Multiphoton objectives

Working distance
Numerical Aperture

\[ NA = (n)\sin(\mu) \]

(a) \( \mu = 7^\circ \) \( NA = 0.12 \)
(b) \( \mu = 20^\circ \) \( NA = 0.34 \)
(c) \( \mu = 60^\circ \) \( NA = 0.87 \)

High NA + Long WD = expensive objective
Multiphoton detectors - NDD

Using the "long way" gives more flexibility, the confocal filter-free scanhead can be finetuned what range of light shall be collected, but the way is long (equals 32 cm glass!) and hence light is lost...

Using the NDDs as "short cuts" avoids loss of light. NDDs filter light via "old days" filtercubes and therefore lack in flexibility.

each NDD houses 2 emission filtercubes
Multiphoton detectors - NDD

Loss of emission light: NDD vs LSMD I

Alexa 488, MaiTai 780nm, 5% (quite high), spectral range emission 500-550nm, no/open pinhole, digital gain etc for NDD (no over/under exposure)
**Multiphoton detectors - GaAsP**

With the very sensitive GaAsP detector right behind the objective we are able to collect more light from weakly fluorescent specimen (higher signal to noise ratio)

- one detector with no filter
- no distinction between different fluorophores...
- Efficience 40 % for 400 - 700 nm

Loss of emission light: NDD vs GaAsP
Bring back home the photons - summary

FL emission is shorter in \( \lambda \) and gets more scattered and dispersed than NIR Ex light

- Loss of emission light i.e. signal light

Light gets lost via the optical pathways

- Loss of emission light i.e. signal light

To compensate this loss
Detectors should have
- better sensitivity
- proximity to specimen
- more

NDD
Keep in mind...

A multiphoton microscope gives you the opportunity to get images from **deep** (e.g. 500 nm) within **living** tissue, whilst **photodamaging only the imaged volume**.

A Multiphoton microscope is a point scanning system which **excites fluorophores within the Focus volume only**. Therefore you collect emission light from this volume only, enabling you to acquire optical slices, **without the use of confocal pinholes**.

Beside this, one is able to **photomanipulate tissue/cells within a very small volume.**
# Comparison of CLSM and 2P

<table>
<thead>
<tr>
<th></th>
<th>LSM</th>
<th>Multiphoton</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>light source</strong></td>
<td>laser UV to VIS</td>
<td>tuneable 50-100fs pulsed IR laser</td>
</tr>
<tr>
<td><strong>depth of visualization</strong></td>
<td>up to 100 µm depending on tissue</td>
<td>up to 1000 µm depending on tissue</td>
</tr>
<tr>
<td><strong>XYZ resolution</strong></td>
<td>via focal plane of objective, pinhole and wavelength</td>
<td>Similar (or worse) as LSM, No pinhole needed</td>
</tr>
<tr>
<td><strong>volume of excitation</strong></td>
<td>throughout the Illuminated tissue</td>
<td>only the focal plane</td>
</tr>
<tr>
<td><strong>sensibility</strong></td>
<td>Loss of signals via optics &gt; Descanned detectors</td>
<td>Enhance signal by use of &gt; Non-descanned detectors</td>
</tr>
</tbody>
</table>
A method called Optical Clearing is available making visualization depth of e.g. 1 mm possible using light of 300 – 633 nm for excitation.
Optical clearing

Removing the optical barriers (different RIs) makes the object invisible – transparent

- Left: water ($n=1.3$) and glass rod ($n=1.5$)
- Right: oil ($n=1.5$) and glass rod ($n=1.5$)
Optical clearing

**Problem:**
Biological tissue: poor light transmission due to interface lipid:water (PM:in/ex-cellular fluids)

**Solution:**
Replace aqueous fluids with solvents which matches Refractive Index (RI) of lipids.
- Penetration of light into the tissue increases,
- Scattering of light decreases.

Optical Clearing Agents (OCAs): aromatic hydrocarbons
- water insoluble but soluble in EtOH or MetOH.
- each clearing is preceded by dehydration (Et/MetOH)
- benzyl-alcohol-benzoate (BABB) (excellent)
- Methyl salycylate (wintergreen oil) (very good)
- Thiodiethanol (TDE) (good)
- Glycerin (poor clearing)
- OCAs have usually a refractive index of around 1.5, hence matching RI of glass, and immersion oil.

ClearT: a detergent- and solvent-free clearing method for neuronal and non-neuronal tissue; http://dev.biologists.org/content/140/6/1364/F1.expansion.html
Optical clearing – go deeper

THANKS FOR YOUR ATTENTION!

Ant head autofluorescence © BioVis – Matyas Molnar