Principles and In Vivo Applications of Two-Photon Microscopy

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Generic Two-Photon Microscope Setup

Scanning

Fluorescence excitation

Image formation and resolution

Fluorescence detection
Two-Photon Fluorescence Excitation

- several photons are virtually simultaneously absorbed, each providing only part of the energy for the molecular transition
- for 2-photon excitation: long wavelength excitation light typically in the near-infrared (NIR) range (700-1000 nm)

\[ S \propto I^2 \]

Using NIR light to create visible light

From wikipedia
Other Nonlinear Interactions

Two-Photon Fluorescence Excitation

• several photons are virtually simultaneously absorbed, each providing only part of the energy for the molecular transition
• for 2-photon excitation: long wavelength excitation light typically in the near-infrared (NIR) range (700-1000 nm)
• non-linear dependence on excitation intensity

$S \propto I^2$

SHG  second-harmonic generation
CARS  coherent Anti-Stokes Raman scattering
2-Photon Excitation: History

1931 Über Elementarakte mit zwei Quantensprüngen. Annalen der Physik 9

Maria Göppert-Mayer (1906-1972), Nobel prize 1963

1960 Invention of the laser (Maiman)
1961 2PE of fluorescence CaF$_2$:Eu$^{2+}$ crystal (Kaiser and Garret)

1990 2-photon laser scanning microscopy (Denk, Strickler, Webb)

Increasing 2P Absorption

- Problem: the probability of a 2-photon absorption event (the „2-photon cross-section“) is very small

- How do we get around this problem:

  1) increase absorption cross sections
     - find/develop appropriate dyes

  2) increase peak intensity
     - concentration in space (focusing)
     - concentration in time (pulsed excitation)
Multiphoton Cross-section

\[
\sigma_j \approx 10^{-16}...10^{-17} \text{ cm}^2 \\
\tau_j \approx 10^{-15}...10^{-16} \text{ s}
\]

Definition of physical unit:

\[
1 \text{ Göppert-Mayer} = 1 \text{ GM} = 10^{-50} \text{ cm}^4 \text{ s/photons}
\]

2-photon Cross-sections

<table>
<thead>
<tr>
<th>2PA $\sigma_2 = \sigma_{ij} \sigma_{jr} \tau_j$</th>
<th>3PA $\sigma_3 = \sigma_{ij} \sigma_{jk} \sigma_{kf} \tau_j \tau_k$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Order of magnitude</td>
<td></td>
</tr>
<tr>
<td>$10^{-40} \text{ cm}^4 \text{s/photons}$</td>
<td>$10^{-82} \text{ cm}^6 \text{s}^2/\text{photons}^2$</td>
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</table>

BM  p-bis(o-methylstyryl)benzene;  
DP (DAPI not DNA-bound) 40,6-diamidino-2-phenylindole, dihydrochloride;  
DN (dansyl) 5-dimethylaminonaphthalene-1-sulfonylhydrazine;  
PY 1,2-bis-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine;  
IC indo-1 with Ca$^{2+}$;  
IF indo-1 without Ca$^{2+}$;  
CG calcium green-1 with Ca$^{2+}$;  
CO calcium orange with Ca$^{2+}$;  
CC calcium crimson with Ca$^{2+}$;  
F3 fluo-3 with Ca$^{2+}$. 

Chris Xu et al.
Excitation Spectra: 1-photon vs. 2-photon

2-photon excitation spectra are similar but not identical to scaled versions of single-photon excitation spectra.

Xu et al. 1996

Increasing Peak Intensity

- increase peak intensity
  - concentration in space (focusing)
  - concentration in time (pulsed excitation)

Pulsed excitation

Intensity

time

$10^{-13}$ s

Focusing

Objective

40x

NA 1.0
Advantage of Pulsed Excitation

$S \propto I^2$

Ex intensity signal $g^{(2)}$

\[ g^{(n)} = \frac{g_p^{(n)}}{(\tau f_R)^{n-1}} \]

- $\tau$ pulse width
- $f_R$ repetition rate
- $n$ number of photons

Typical values (Ti:sapphire laser):

$100 \text{ fs} = 10^{-13} \text{ s}$ pulse width

$100 \text{ MHz} = 10^8 \text{ s}$ repetition rate

$g^{(2)} \approx 10^5$
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Two-Photon Microscope Setup

Laser

Titanium:sapphire laser
(700-1000 nm)
100 fs pulse width
100 MHz repetition rate

Custom-built Laser-Scanning Microscope
Special lasers for 2PE

- high peak energies needed for excitation
  - mode-locked pulsed laser
    (~100 fs pulse width, 80 MHz, 2-3 W average output)
  - Titanium:Sapphire laser
    (about 700-1060 nm)


Pulse broadening due to dispersion
Dispersion compensation

- provide **negative dispersion** using combination of prisms or gratings

![Grating pair](image1) ![Multi-Prism-Array](image2)

Scanner types

- **Galvanometric mirrors**
  - up to 1 kHz line rate
- **Resonant mirrors**
  - up to 12 kHz
- **Acousto-optical deflectors (AOD)**
  - up to 100 kHz, random access mode
- **Multifocal beam excitation**
  - parallelization
Intraenal optical sectioning (3D resolution)

Optical sectioning

Bleaching experiment: Denk et al, 1995

by Werner Göbel
How about the spatial resolution?

• Resolution is determined by width of Point Spread Function (PSF)

Confocal

\[ PSF_{\text{confocal}} = PSF_{\text{illumination}} \cdot PSF_{\text{detection}} \approx PSF^2(v, u) \quad \lambda \approx 400-500 \text{ nm} \]

2-Photon

\[ PSF_{2-\text{photon}} = (PSF_{\text{illumination}})^2 \approx PSF^2(v/2, u/2) \quad \lambda \approx 900 \text{ nm} \]

(2-Photon confocal)

\[
(PSF_{2-\text{photon,confocal}} = (PSF_{\text{illumination}})^2 \cdot PSF_{\text{detection}} \approx PSF^2(v/2, u/2) \cdot PSF(v, u))
\]

Comparison of PSFs

Calculated PSFs (NA=1.3)

[Images of PSFs for different conditions]

Jonkman & Stelzer
Comparison of PSFs

Integrated intensities of the PSFs as a function of depth

2-photon microscopy does NOT provide an improved spatial resolution.

So what is its real advantage?
The Scattering Problem

Typical scattering lengths:
30-50 μm visible, 200 μm NIR

Fluorescence Detection

Two-photon detection strategy
„You know where the fluorescence light is coming from, therefore collect as much as you can!“
Comparison to Confocal Microscopy

Denk, J. Biomed. Imaging 1996

Options for Detection

Denk et al. 1995
Maximize fluorescence detection in brain slice experiments

Supplementary fiber-optic fluorescence collection (SUFICS)

Engelbrecht, Göbel, Helmchen Optics Express 2009
A significant gain in signal

with 40x objective

Supplementary fiber-optic fluorescence collection (SUFICS)
Summary 1: Principles of Two-Photon Microscopy

- 2-photon excitation is a non-linear process
- High photon densities – using pulsed lasers and spatial focusing – increase 2PE
- 2PE provides intrinsic optical sectioning
- A simple "maximize detection" strategy applies
- The theoretical spatial resolution is worse than for confocal microscopy
- The real strength of 2-photon microscopy lies in its weaker sensitivity to light scattering

Windows to the Brain

"Open cranial window"

"Thinned skull window"

"Chronic sealed glass window"

Routine imaging depth: up to 400-800 micron
Neural circuit dynamics across multiple time scales

**Electrophysiology**

**Calcium Imaging**

- msec
- sec
- min
- hours
- days
- months
- years

- Reflexes
- Sensory Processing
- Channel kinetics
- Modulation
- Learning
- Plasticity
- Memory
- Forgetting

**Spike activity**

8 trials

200 ms

The standard: Mechanical laser scanning

Two-photon microscope

- Laser
- x-y Scan-mirrors
- Fluorescence detection
- 2D or 3D

**Typical frame rates:** 1-10 Hz
Even with resonant scanners: 30 Hz

- xy: Galvanometric scan mirrors
- z: Piezoelectric focusing device

25 μm
In vivo population loading with calcium indicators

Targeted Recording

% ΔF/F

Spontaneous activity during anesthesia

Neuronal population in layer 2/3 of neocortex filled with calcium indicator

"Calcium transient (~ 500 ms)"

ΔF/F "percentage fluorescence change relative to baseline"
Action potential-evoked calcium transients

- Oregon Green BAPTA-1 (Calcium indicator)
- Sulforhodamine 101 (astroglia marker)

Kerr, Greenberg & Helmchen, PNAS 2005

Orientation Tuning in Mouse Visual Cortex

Björn Kampa
Non-mechanical xy-scanning

Grewe et al., Nat Methods 2010

Temporal and spatial dispersion compensation

1. Spatial focus
2. Pulse width
In vivo AOD-image of neuronal population

Layer 2/3 neocortex

- **Oregon Green BAPTA-1** (Calcium indicator)
- **Sulforhodamine 101** (astroglia marker)

High-speed random access scanning

Pattern scanning

- **Point 1**: Laser power ~ 12 μs
- **Point 2**: Laser power ~ 12 μs
- **Cell 1**: Laser power ~ 60 μs
- **Cell 2**: Laser power ~ 60 μs

Benjamin Grewe
Random access pattern scanning (RAPS)

Example: High-speed calcium imaging

55 neurons recorded at 300 Hz
Single spikes can be detected ...

Grewe et al., Nat Methods 2010

Example: responses to natural movies

Response epochs of visual cortex neurons

Björn Kampa
Benjamin Grewe
Neural circuit dynamics across multiple time scales

- Electrophysiology
- Calcium Imaging

msec sec min hours days months years

- Reflexes
- Adaptation
- Sensory Processing
- Plasticity
- Learning
- Development
- Modulation
- Memory
- Forgetting

Channel kinetics

Chronic experiment → Time

Same animal surgery habituation Repeated imaging sessions

Genetically-encoded calcium indicators (GECIs)

1997 Cameleon
PeriCam
Camgaroo
Inverse PeriCam
GCaMP
D3cpv
Yellow Cameleons
TN-XXL
YC-Nanos
...

2011 GECOs
GCaMP5, ...

Single Protein Indicators

Tandem Protein Indicators

FRET effect
Yellow cameleon 3.60

Kotlikoff, J Physiol 2007

Nagai et al. PNAS 2004

AAV-induced expression of YC3.60

with Maz Hasan (Heidelberg) and Sebastian Kügler (Göttingen)

Two-photon imaging after >3 weeks

Lütcke, Murayama et al. Front. Neural Circuits 2010

K_d = 250 nM, n_Hill = 1.7
Chronic window to the brain

Two-photon imaging in superficial cortical layers

5 weeks after implant

Long-term repeated imaging of the same population
Ratiometric calcium measurements using YC3.60

Mouse Neocortex (Layer 2/3)

Simultaneous electrophysiology

Lütcke et al. Front. Neural Circuits 2010

Stable functionality of YC3.60

Action potential sensitivity

YC3.60

6x Speed

20 µm

>3.5 months after window prep
Imaging in awake mice

Setup for calcium imaging in head-restrained awake mice

Cellular signals in barrel cortex

Whisking behavior

Mean whisk angle
Whisking velocity

Example cells
- Active during whisking
- Non-active during whisking

Differential network modulation during whisking

3x Speed

Whisking velocity

5% ΔR/R
1%
Differential network modulation during whisking

Mean whisking angle

Differential network modulation during whisking
Aligned to behavior transitions

Up-modulated cells ('whisking-related')

Down-modulated cells

Example cell

Neuron

Neuron

0.2 eAP/bin

0.2 eAP/bin

Time (s)

Time (s)

0

0

2.5

2.5

quiet

quiet

whisking

whisking

% ΔR/R

% ΔR/R

5

5

0

0

0.5

0.5

5

5

0

0

0.5

0.5

1

1

Up-modulated

Down-modulated

non-modulated

6%

74%

20%
Correlating calcium signals with whisking

Kristina Schulz
David Margolis

Stability of whisking-related modulation

Example cells
- Active during whisking

Whisking velocity
- 3x Speed

5% ΔR/R
1% ΔR/R
Next day ....
Same mouse, same neurons ...

3x Speed

Example cells
○ Active during whisking

Whisking velocity

3 months later ....
Same mouse, same neurons ...

3x Speed

Example cells
○ Active during whisking

Whisking velocity
Stability of state-dependent modulation over weeks and months

Sessions were spread over up to 3 months

Post hoc discrimination of cell types

Langer & Helmchen
Pflügers Archiv, 2011
Discrimination of neuronal cell types

Post hoc immunostaining of GABAergic interneurons

Parvalbumin+

Calretinin+

Dominik Langer

Summary 2

• In vivo two-photon microscopy is a versatile tool for studying various aspects of neural dynamics

• New scanning approaches using AODs enable high-speed measurements of neuronal population activity in vivo

• The emerging use of genetically encoded calcium indicators opens a wide field for long-term longitudinal studies of neural dynamics

• In vivo calcium imaging in behaving mice permits dissection of circuits dynamics related to particular behaviours
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