Multiphoton Deep Imaging of the Mouse Brain

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Deep tissue imaging

Multiphoton endoscopy

Fiber optics
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The difficulty of understanding the brain

1 transistor

\[ E=mc^2 \]

fMRI

10^{11} neurons

Need new tools to build the bridge

\[ \text{a few neurons} \]

\[ 10^8 \text{ neurons} \]
The need for in vivo deep tissue imaging

$10^2$ neurons

$10^5$ to $10^6$ neurons

mouse brain ($10^8$ neurons)

High temporal (> 10 Hz) and spatial (< 1 μm) resolution, deep (> 1 mm), and large scale (> 1 mm$^3$) imaging, within scattering brain tissue, is a major scientific challenging.
Transparent brain is relatively easier.

Camera does not work in scattering sample.

tissue scattering and parallel data acquisition?
Scattering limits high resolution optical imaging

Mouse brain in vivo under a regular light microscope

Think about looking through Tofu or cheese…

Absorption, Scattering, Inhomogeneity

Multiphoton microscopy for deep brain imaging

Denk, Strickler, and Webb 1990
Multiphoton fluorescence excitation

Excited State \( f \)

Intermediate State \( j \)

Ground State \( i \)

One-photon cross section:
\[ \sigma_{if} \sim 10^{-16} \text{ to } 10^{-17} \text{ cm}^2 / \text{photon} \]

Two-photon cross section:
\[ \sigma_2 = \sigma_{ij} \sigma_{jf} \tau_j \sim 10^{-49} \text{ cm}^4 / \text{s} / \text{photon} \quad (or \ 10 \text{ GM}) \]

two-photon signal \( \propto (f \tau)^{-1} \)

Femtosecond pulsed excitation is typically required.
Multiphoton Fluorescence Microscopy
(1) nonlinear and (2) longer wavelength

Fluorescein molecule

1-photon blue excitation

2-photon red excitation

hv

2hv

Green fluorescence

2PE \propto I^2

Denk, Strickler, and Webb 1990
One Photon

Signal $\propto I$

Two Photon

Signal $\propto I^2$

3D scanning of the focal spot to form a 3D image.
Confocal microscopy does not work well in thick scattering sample.

Non-scattering specimen

Multiphoton microscopy works well with scattering sample.

Deep tissue, high resolution imaging is the advantage of MPM.

Scattering specimen
Multiphoton microscopy (MPM) in thick scattering tissue

Nonlinear excitation enables a sharp focus in 3D in a scattering environment.

No inverse scattering is necessary as long as a sharp focus can be formed.
Tissue scattering is the main limitation in deep imaging

Scattering decreases the excitation photons at the focal volume (exponential decay of $S$)

- **Signal-to-noise ratio problem**: intrinsic quantum noise of photons
  - Photon shot noise limit
- **Signal-to-background problem**: no longer able to form a sharp focus
  - Inverse scattering problem (want to avoid)
Signal-to-background ratio in scattering tissue

$SBR \sim \frac{\text{Signal}}{\text{Background}} \sim z^2 \exp(-2z/l_s)$


**Strategy:** reduce scattering AND increase excitation confinement
long wavelength for reducing excitation attenuation in brain

excitation power $\propto e^{-z/l_a} e^{-z/l_s}$

effective attenuation length $= \frac{1}{1/l_a + 1/l_s}$

imaging depth $\propto \frac{1}{1/l_a + 1/l_s}$

Optimum wavelength is a trade-off between absorption and scattering. 1300 nm and 1700 nm windows.

Nature Photonics, 7, 205–209, 2013
Fluorophores for the long wavelength spectral windows

Fluorophores for 2-photon excitation at 1300 nm and 1700 nm ~ very few!

Long wavelength is not practical for 2-photon excitation.
Fluorophores for the long wavelength spectral windows

Fluorophores for two-photon excitation at 1300 nm and 1700 nm ~ very few!

What about three-photon excitation (3PE)?
Fluorophores for the 1300 and 1700 nm spectral windows for 3-photon excitation

All orange and red dyes for 3PE at ~ 1700 nm.

<table>
<thead>
<tr>
<th>Fluorescent proteins</th>
<th>1PE (nm)</th>
<th>Fluorescence emission</th>
<th>Possible 3PE (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orange (mOrange, mKO)</td>
<td>548</td>
<td>560</td>
<td>1645</td>
</tr>
<tr>
<td>tdTomato, Ds-red</td>
<td>555</td>
<td>583</td>
<td>1665</td>
</tr>
<tr>
<td>Red (mCherry, J-red)</td>
<td>585</td>
<td>610</td>
<td>1755</td>
</tr>
<tr>
<td>Far-red</td>
<td>590</td>
<td>650</td>
<td>1770</td>
</tr>
</tbody>
</table>

3PE of blue and green fluorophores, GFPs, and GCaMPs at 1300 nm.

Long wavelength spectral windows

3-photon excitation
3-photon excitation reduces out-of-focus background, and improves the 3D confinement.

Axial fluorescence profile in non-scattering sample

\[ \text{Signal } \propto I^2 \]
3PE provides better 3D confinement in scattering tissue.

Better 3D confinement leads to better tissue penetration.
3-photon imaging has vastly improved SBR for deep imaging in non-sparsely labeled brain.

Fluorescein labeled brain vasculature
2P at 920 nm 3P at 1300 nm

Transgenic mouse with GCaMP6s
2P at 920 nm 3P at 1300 nm

Between 700 and 800 µm:
SBR for 2P < 1.0

At 1060 µm depth (below the white matter):
SBR for 3P > 20

Manuscript in preparation
In vivo 3-photon imaging of red fluorescent protein (RFP) labeled neurons beyond white matter

Maximum power at the sample is ~ 22 mW.
In vivo 3-photon imaging of RFP neurons beyond white matter

Brainbow mouse

fluorescence of neurons

third harmonic generation of myelinated axons

SP at ~1.1 mm

CLEO 2012, postdeadline paper, CTh5C.4.
Nature Photonics, 7, 205–209, 2013
In vivo imaging through unthinned, intact mouse skull

3-photon imaging at 1700 nm

Green: THG  Red: fluorescence

Manuscript in preparation
In vivo imaging of brain function

- Ca-imaging for recording neuronal activity in vivo
  - Organic dyes
  - Genetically engineered fluorescent protein probes

- Green fluorescence protein Ca-probes (GCaMPs) (3-photon excitation at 1300 nm)
- Red fluorescent protein based Ca-probes (RCaMP) (3-photon excitation at 1700 nm)
Imaging spontaneous activity of deep L6 neurons
GCaMP6 at 1300 nm
(single trial measurement)

10 min movie, sped up by 60x

THG + GCaMP fluorescence

FOV = 300 µm x 300 µm
Frame Rate = 8.49Hz
Frame Size = 256 x 256 pixels
Mouse: 16 weeks old
Power = 36 mW at 800kHz
(Newport Spirit-NOPA)
Low pass filtered
\[\Delta F/F \text{ vs time}\]

Raw data in photons/neuron/frame
(recorded at 8.49 frames/s)
Imaging the brain activity of an entire mouse cortical column (Somatosensory Cortex L1 to L6 of a densely labeled transgenic mouse)

Field-of-view (FOV) 235 µm at 8.49 frames/s
Power: 4.1 mW at 130 µm and 20.5 mW at 800 µm
Green: Fluorescence
Magenta: Third Harmonic Generation (THG)

CamKII-tTA/GCaMP6s
Densely labeled transgenic mouse provided by Tolias and Reimer at Baylor Medical College
3-photon imaging of spontaneous activity in hippocampus within an intact mouse brain

Imaging the hippocampus is much harder because the white matter is much more scattering than the grey matter (appears to be ~ 2.5x)!!

Scattering length in mouse brain at 1700 nm

- $l_e \approx 366 \mu m$ for grey matter
- $l_e \approx 140 \mu m$ for white matter
- $l_e \approx 310 \mu m$ for hippocampus
In vivo imaging of hippocampal neurons within an intact mouse brain
Using 3-photon excitation of GCaMP6s at 1300 nm

Purple = Third harmonic generation
Green = GCaMP6s fluorescence
FOV = 400 µm x 400 µm
(Newport Spirit-NOPA)

**Imaging spontaneous activity in hippocampus within an intact mouse brain (Single trial measurement)**

3-photon excitation of GCaMP6s at 1300 nm

Depth = 984 µm

Watch mouse thinking, deeply

17-week old mouse
FOV = 200 µm x 200 µm
Frame rate = 8.49 Hz
power ~ 50 mW at 800 KHz repetition rate

Imaged the same mouse multiple times over a period of several weeks.

Activity imaging of mouse hippocampus (single trial measurement)

Raw data in photons/neuron/frame (recorded at 8.49 frames/s)

Watch mouse thinking, deeply and quantitatively.

Nature Methods, in press
Activity imaging in hippocampus

High spatial resolution and contrast: nucleus exclusion is clearly visible.
How to go forward from here?

(1) Excitation source (2x to 3x)
(2) Adaptive optics
(3) Other possibilities
Adaptive optics (AO) may be the low hanging fruit.

AO should have larger impact for deep imaging.
AO should have larger impact for 3-photon imaging.
Impact of adaptive optics (AO) on multiphoton imaging

AO correction for the same 2-m focal length cylindrical lens at objective back pupil

AO has order(s) of magnitude more impact for higher-order excitation!

**AO for in vivo 3-photon imaging of RFP-labeled neurons at 1000 µm depth**

Low-order correction provides a large field-of-view.
Correction phase maps at different imaging depths

Correcting aberration caused by brain tissue, not by imaging system.
We are pushing the depth limit using AO for 3P.  
1700 nm 3PE of RFP-labeled neurons in Brainbow mouse at 1530 µm depth.

AO should have larger impact for deep imaging.  
AO should have larger impact for 3-photon imaging.  
But longer wavelength reduces the AO impact…

Manuscript in preparation
But what are we compensating?

Aberration  x  x  scattering

RFP fluorescence from Brainbow mouse

Perhaps easier and more practical.
What else?

Only imaging the neurons (~10x)

But how to do it?

What about motion in vivo?

With the optimized laser, AO, smart scanning,
~2 orders of magnitude improvement seems possible.
(~within a few years)

100x more neurons OR another ~400 µm in depth.

How to go beyond ~2 mm in mouse brain???
The dream

Really difficult:
increase excitation cross sections of fluorescent probes
Need chemist and protein engineers, but have been tried for the last 20 years

Brave new world:
Tissue clearing of living brain with adaptive illumination

Tissue clearing of living brain using clearing materials

Absorption limit: \[ \mu_{\text{eff}} = \sqrt{3\mu_a (\mu'_s + \mu_a)} \approx \sqrt{3\mu_a \mu'_s} > 0.5 \text{ mm}^{-1} \]
Pushing the Limits of Optical Brain Imaging

• Long wavelength and 3-photon imaging is superior for deep penetration.
  – 3-photon microscopy of hippocampal structure and function
  – Through skull imaging

Particularly suited for deep and densely labeled volume imaging

• Improvement of orders magnitude may be possible
  – Optimization of excitation source (2x to 3x)
  – Adaptive optics (~ 5x)
  – Smart scanning (~10x)

  – Increase probe performance (cross section, wavelength, etc.), tissue clearing with chemical or with smart light…

Increasing difficulty

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