In vivo Two-photon Imaging

Ninglong Xu, 2014.10, ION Happy hour
Talk outline:

1. Two-photon imaging basic theory.
   - \( \text{Ca}^{2+} \) indicators.
   - Quantification of fluorescent signals over time.
   - In vivo application: Imaging dendrites, somata and axons during behavior.
4. Comparison with electrodes.
5. Comparison with other imaging methods.
Two-photon fluorescence imaging
Two-photon fluorescence imaging

A Spatial compression of photons by objective lens

B Temporal compression of photons during femtosecond pulses

Continuous laser

Femtosecond-pulsed laser

From http://parkerlab.bio.uci.edu
Two-photon scanning microscopy

Confocal VS Two-photon
Scattering tissue: Confocal vs Two-photon

Confocal

2-Photon

Scattered emission blocked by pinhole.
Application 1:
in vivo structural imaging
Long-term chronic imaging

**Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex**

Joshua T. Trachtenberg*,†, Brian E. Chen*,†, Graham W. Knott‡, Guoping Feng§, Joshua R. Sanes§, Egbert Welker‡ & Karel Svoboda*

* Howard Hughes Medical Institute, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724, USA
‡ Institut de Biologie Cellulaire et de Morphologie, Université de Lausanne, Rue du Bagnon 9, CH-1005, Lausanne, Switzerland
§ Department of Anatomy and Neurobiology, Washington University School of Medicine, St Louis, Missouri 63110, USA
† These authors contributed equally to this work
Application 2: in vivo functional imaging
Sources of Ca\textsuperscript{2+} signal

Main contributors to neuronal Ca\textsuperscript{2+} signaling:

- Voltage-Gated Calcium Channels
- NMDA receptors
- Calcium-permeable AMPA receptors
- Intracellular calcium stores
Dendritic spines as basic functional units of neuronal integration

Rafael Yuste & Winfried Denk*

Biological Computation Research Department, AT&T Bell Laboratories, Murray Hill, New Jersey 07974, USA
* To whom correspondence should be addressed

- Isolated Ca signals in spines - synaptic input
- Spine heads contain VSCC - AP induced signals.
- NMDAr and AMPAr required for SYN spine signals.
- Coincidence detection in spines!

Line scan: 2 ms / line (500 Hz)
Ca$_2^+$ Indicators

**Chemical calcium indicator**

- **Ca$_2^+$-chelating site**
- **Fluorophore**
- **Fura-2**

**FRET-based GECI**

- **440 nm**
- **ECFP**
- **Venus**
- **Ca$_2^+$**
- **530 nm**

**Single-fluorophore GECI**

- **485 nm**
- **EGFP**
- **Ca$_2^+$**
- **515 nm**

**Organic molecules**

- **Fluorescence resonance energy transfer (FRET)**
- **Ratiometric**
- **ΔR/R**

- **Non-ratiometric**
- **ΔF/F**

---

Grienberger & Konnerth, Neuron, 2013
response kinetics but it does not show reliable single action-

Recently, after elucidating the structure of the GCaMP2, and calretinin (Baimbridge et al., 1992). In calcium imaging in equilibrium with the calcium ions that are bound to endoge-

the cytosolic free calcium concentration. Free calcium ions are aware of the fact that calcium indicators measure changes in

indicators, including some representative references and exam-

in the emitted fluorescence (Nakai et al., 2001; Tian et al., 2009).

calcium, calmodulin-M13 interactions elicit conformational

Camgaroo 2 Drosophila, mouse olfactory bulb Yu et al., 2003; Hasan et al., 2004

Camgaroo 1 Drosophila Yu et al., 2003

Fura-2 140 Mouse neocortex Sohya et al., 2007

Indo-1 230 Mouse neocortex Stosiek et al., 2003

Fluo-4 345 Mouse neocortex, Xenopus larvae Sato et al., 2007; Demarque and Spitzer, 2010

Rhod-2 570 Mouse neocortex, Zebrfish Takano et al., 2006; Yaksi et al., 2009

X-rhod-1 700 Mouse neocortex Nagayama et al., 2007

<table>
<thead>
<tr>
<th>Name</th>
<th>Kd (nM)</th>
<th>Examples of In Vivo Applications</th>
<th>Representative References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oregon Green BAPTA-1</td>
<td>170</td>
<td>Mouse neocortex, mouse hippocampus, mouse olfactory bulb, rat neocortex, rat cerebellum, ferret neocortex, cat neocortex, zebrafish</td>
<td>Dombeck et al., 2010; Sullivan et al., 2005; Ohki et al., 2005; Li et al., 2008; Greenberg et al., 2008; Rochefort et al., 2011; Sumbre et al., 2008; Wachowiak et al., 2004</td>
</tr>
<tr>
<td>Calcium Green-1</td>
<td>190</td>
<td>Mouse neocortex, mouse olfactory bulb, honeybee, turtle, zebrafish, rat neocortex</td>
<td>Dombeck et al., 2009; Oka et al., 2006; Galizia et al., 1999; Wachowiak et al., 2002; Brustein et al., 2003; Svoboda et al., 1997</td>
</tr>
<tr>
<td>Fura-2</td>
<td>140</td>
<td>Mouse neocortex</td>
<td>Sohya et al., 2007</td>
</tr>
<tr>
<td>Indo-1</td>
<td>230</td>
<td>Mouse neocortex</td>
<td>Stosiek et al., 2003</td>
</tr>
<tr>
<td>Fluo-4</td>
<td>345</td>
<td>Mouse neocortex, Xenopus larvae</td>
<td>Sato et al., 2007; Demarque and Spitzer, 2010</td>
</tr>
<tr>
<td>Rhod-2</td>
<td>570</td>
<td>Mouse neocortex, Zebrfish</td>
<td>Takano et al., 2006; Yaksi et al., 2009</td>
</tr>
<tr>
<td>X-rhod-1</td>
<td>700</td>
<td>Mouse neocortex</td>
<td>Nagayama et al., 2007</td>
</tr>
</tbody>
</table>

**Table 1. Frequently Used Fluorometric Calcium Indicators**

<table>
<thead>
<tr>
<th>Name</th>
<th>Kd (nM)</th>
<th>Examples of In Vivo Applications</th>
<th>Representative References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chemical Calcium Indicators</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oregon Green BAPTA-1</td>
<td>170</td>
<td>Mouse neocortex, mouse hippocampus, mouse olfactory bulb, rat neocortex, rat cerebellum, ferret neocortex, cat neocortex, zebrafish</td>
<td>Dombeck et al., 2010; Sullivan et al., 2005; Ohki et al., 2005; Li et al., 2008; Greenberg et al., 2008; Rochefort et al., 2011; Sumbre et al., 2008; Wachowiak et al., 2004</td>
</tr>
<tr>
<td>Calcium Green-1</td>
<td>190</td>
<td>Mouse neocortex, mouse olfactory bulb, honeybee, turtle, zebrafish, rat neocortex</td>
<td>Dombeck et al., 2009; Oka et al., 2006; Galizia et al., 1999; Wachowiak et al., 2002; Brustein et al., 2003; Svoboda et al., 1997</td>
</tr>
<tr>
<td>Fura-2</td>
<td>140</td>
<td>Mouse neocortex</td>
<td>Sohya et al., 2007</td>
</tr>
<tr>
<td>Indo-1</td>
<td>230</td>
<td>Mouse neocortex</td>
<td>Stosiek et al., 2003</td>
</tr>
<tr>
<td>Fluo-4</td>
<td>345</td>
<td>Mouse neocortex, Xenopus larvae</td>
<td>Sato et al., 2007; Demarque and Spitzer, 2010</td>
</tr>
<tr>
<td>Rhod-2</td>
<td>570</td>
<td>Mouse neocortex, Zebrfish</td>
<td>Takano et al., 2006; Yaksi et al., 2009</td>
</tr>
<tr>
<td>X-rhod-1</td>
<td>700</td>
<td>Mouse neocortex</td>
<td>Nagayama et al., 2007</td>
</tr>
<tr>
<td><strong>Genetically Encoded Calcium Indicators</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Camgaroo 1</td>
<td></td>
<td>Drosophila</td>
<td>Yu et al., 2003</td>
</tr>
<tr>
<td>Camgaroo 2</td>
<td></td>
<td>Drosophila, mouse olfactory bulb</td>
<td>Yu et al., 2003; Hasan et al., 2004</td>
</tr>
<tr>
<td>Inverse pericam</td>
<td>200</td>
<td>Zebrafish, mouse olfactory bulb</td>
<td>Hasan et al., 2004; Li et al., 2005</td>
</tr>
<tr>
<td>GCaMP 2</td>
<td>840</td>
<td>Mouse olfactory bulb, mouse cerebellum</td>
<td>Fletcher et al., 2009; Diez-Garcia et al., 2005</td>
</tr>
<tr>
<td>GCaMP 3</td>
<td>660</td>
<td>Mouse neocortex, mouse hippocampus, Drosophila, C. elegans</td>
<td>Tian et al., 2009; Dombeck et al., 2010; Seelig et al., 2010; Tian et al., 2009</td>
</tr>
<tr>
<td>Yellow Cameleon 3.6</td>
<td>250</td>
<td>Mouse neocortex</td>
<td>Lütcke et al., 2010</td>
</tr>
<tr>
<td>Yellow Cameleon Nano</td>
<td>15–50</td>
<td>Zebrafish</td>
<td>Horikawa et al., 2010</td>
</tr>
<tr>
<td>D3cpV</td>
<td>600</td>
<td>Mouse neocortex</td>
<td>Wallace et al., 2008</td>
</tr>
<tr>
<td>TN-XL</td>
<td>2200</td>
<td>Drosophila, macaque</td>
<td>Mank et al., 2006; Heider et al., 2010</td>
</tr>
<tr>
<td>TN-L15</td>
<td>710</td>
<td>Mouse neocortex</td>
<td>Heim et al., 2007</td>
</tr>
<tr>
<td>TN-XXL</td>
<td>800</td>
<td>Drosophila, mouse neocortex</td>
<td>Mank et al., 2008; Mank et al., 2008</td>
</tr>
</tbody>
</table>

Kₐ dissociation constant in nM. Kₐ values taken from The Molecular Probes Handbook (chemical calcium indicators), Nagai et al., 2001 (Pericam), Tian et al., 2009 (GCaMP), Nagai et al., 2004 (YC 3.6), Horikawa et al., 2010 (YC-Nano), Palmer et al., 2006 (D3cpv), and Mank et al., 2008 (TN-based).

<table>
<thead>
<tr>
<th>Sensor</th>
<th>Dynamic range (Fₘₐₓ/Fₘₜₜₜ)</th>
<th>Kₐ (nM)</th>
<th>Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCaMP3</td>
<td>13.5±0.7</td>
<td>345±17</td>
<td>2.54±0.04</td>
</tr>
<tr>
<td>GCaMP5G</td>
<td>45.4±0.9</td>
<td>447±10</td>
<td>2.46±0.04</td>
</tr>
<tr>
<td>GCaMP6s</td>
<td>63.2±3.1</td>
<td>144±4</td>
<td>2.90±0.17</td>
</tr>
<tr>
<td>GCaMP6m</td>
<td>38.1±1.8</td>
<td>167±3</td>
<td>2.96±0.04</td>
</tr>
<tr>
<td>GCaMP6f</td>
<td>51.8±2.8</td>
<td>375±14</td>
<td>2.27±0.10</td>
</tr>
</tbody>
</table>
Concentration is essential for the interpretation of the results. This decision should be guided by the scientific goals of the measurement and by the cells of interest. For example, fluorescent signals recorded with low-affinity indicators, which add little buffer capacity to the cell, reflect more accurately the change in the free cytosolic calcium concentration. These calcium signals will have faster rise and decay times than those recorded with high-affinity indicators (Helmchen et al., 1997). However, the use of low-affinity calcium indicators is limited by the need for sufficient sensitivity. This problem becomes increasingly significant when imaging in the noisy in vivo condition and when imaging small structures, such as dendritic spines. In these conditions, high-affinity calcium dyes remain, with all their limitations, the indicators of choice. Fortunately, calcium indicators with different properties can often be easily used complementarily in an experimental series. The new developments will certainly add up to our ability of deciphering the highly complex mechanisms of neuronal signaling in the intact nervous system.

Dye-Loading Approaches

The loading of calcium indicators into neurons depends on the type of calcium indicator, the biological preparation, and the specific scientific question. Figure 3A illustrates the three most widely used approaches for dye loading of individual neurons. In the early imaging experiments, chemical calcium dyes were delivered through sharp microelectrodes both in vitro (Jaffe et al., 1992) and in vivo (Svoboda et al., 1997) (Figure 3A, left panel). In more recent years, dye delivery through whole-cell patch-clamp micropipettes became the standard procedure for single-cell dye loading for many applications (Figure 3A, middle panel) (Eilers and Konnerth, 2009; Margrie et al., 2002). A particularly useful variant of this method involves in vivo whole-cell recordings that are performed under visual guidance using two-photon imaging by applying the “shadow patching” technique (Jia et al., 2011; Kitamura et al., 2008). This approach can be combined with the targeting of genetically identified cells expressing a fluorescent marker protein (Margrie et al., 2003).

Figure 3. Dye-Loading Approaches

(A) Single-cell loading by sharp electrode impalement (left panel), whole-cell patch-clamp configuration (middle panel), and single-cell electroporation (right panel). Note that these approaches can be used for loading of chemical and genetically encoded calcium indicators.

(B) “Acute” network loading. Many neurons are labeled simultaneously by acetoxymethyl ester (AM) loading (left panel), by loading with dextran-conjugated dye (middle panel), and by bulk electroporation (left panel).

(C) Expression of genetically encoded calcium indicators (GECI) by viral transduction (left panel), in utero electroporation (middle panel), and generation of transgenic mouse lines (right panel).
How to load the indicators into cells

• **Bolus loading with OGB-1-AM-ester:**
  • Pros:
    • OGB-1 is still a golden standard for measuring intracellular $[\text{Ca}^{2+}]$.  
    • Good loading efficiency, does not depend on virus quality.
  • Cons:
    • Non-selective, load both neurons and glia
    • More invasive: DMSO can be toxic.
    • Cannot do chronic imaging, staying in cells for only a few hours.
How to load the indicators into cells

Using GECIs

• **Expression with virus**
  • Most widely used, can be very specific (location and cell-type), can do chronic imaging.
  • Long-time expression could be toxic; require good virus prep.

• **In Utero electroporation/virus injection**
  • Good for labeling L2/3 pyramidal neurons, useful if you don’t have good virus.
  • Low success rate for beginners.

• **Transgenic mice**
  • Cell-type specific; less toxic issue; No injection.
  • Limited availability of animals; Slow breeding; expensive.
Quantification of Ca$^{2+}$ signals

1. Define an ROI (region of interest).
2. Average grey values of all pixels in the ROI, $F$.
3. Define a background ROI (with no cell), $F_{\text{background}}$, and subtract background from signal (optional).
4. Define baseline $F_0$.
5. $\Delta F/F = (F - F_0)/F_0$
ROI selection

Morphology-based

Activity-based

Two-step Movement Correction

1. Inter-frame movement
2. Intra-frame movement

ROI alignment across imaging sessions

Imaging session 3

Huber et al, Nature 2012
Two-photon fluorescence imaging in behaving animals

**Advantages over electrodes**

- High spatial resolution – single cell and subcellular recordings.
- High throughput – record hundreds of neurons simultaneously.
- Reveals spatial organization.
- Long-term chronic imaging.
- Genetically targetable.

**Limitations**

- Temporal resolution.
- Sensitivity only for suprathreshold.
- Integration with optogenetics to be worked out.
Spatial resolution: subcellular recording

In vivo dendritic calcium dynamics in neocortical pyramidal neurons

Karel Svoboda, Winfried Denk, David Kleinfeld & David W. Tank

Biological Computation Research Department, Bell Laboratories, Lucent Technologies, Murray Hill, New Jersey 07974, USA
The first success in population imaging in vivo: The bolus loading technique

Membrane-permeant indicators:
Calcium green-1 acetoxymethyl (AM) ester, Fura-2 AM, Fluo-4 AM, and Indo-1 AM.

Later:
Oregon Green BAPTA-1 (OGB-1 AM)

Christoph Stosiek, ... Arthur Konnerth, PNAS 2003
Functional imaging with cellular resolution reveals precise micro-architecture in visual cortex

Kenichi Ohki, Sooyoung Chung*, Yeang H. Ch'ng*, Prakash Kara* & R. Clay Reid

Department of Neurobiology, Harvard Medical School, Boston, Massachusetts 02115, USA
* These middle authors contributed equally to this work

Ohki et al, Nature 2005
Using OGB-1 in behaving animal

Limitation:
Only one imaging session

Komiyama et al, Nature 2010
Genetically encoded Ca\textsuperscript{2+} indicators

1. Long-term chronic imaging.
2. Genetically targetable.
Subcellular Ca2+ imaging in behaving mouse

Xu et al., *Nature* 2012
Imaging activity in axonal projections

Petreanu et al, Nature 2012
Supplementary Figure 16 | Spatially intermingled representations. Spatial locations of classified neurons. Individual regions of interest (ROIs) colored according to the scale in a. ROIs with yellow boundaries correspond to non-classified, task-related neurons. Numbers below images denote the spatial clustering index (SCI; > 1, clustering; 1, unclustered) and the p-value of clustering. We measured the center coordinates of each active cell (including active but unclassified cells). SCI is the ratio of the mean distance between neuronal pairs belonging to different representations divided by the mean distance between neuronal pairs belonging to the same representations exclusively. Under the null hypothesis that different representations are intermingled, SCI ~ 1. SCI > 1 indicates spatial clustering. To test for statistical significance we shuffled the cell's labels and computed SCI on the shuffled data 1000 times; the p-value is the fraction of shuffled SCI higher than the unshuffled SCI. Significant clustering was not detected.
In vivo 2-photon Ca\textsuperscript{2+} imaging

Advantages over electrodes

\begin{itemize}
\item High spatial resolution – single cell and subcellular recordings.
\item High throughput – record hundreds of neurons simultaneously.
\item Reveals spatial organization.
\item Long-term chronic functional imaging.
\item Genetically targetable.
\end{itemize}

Limitations

\begin{itemize}
\item Temporal resolution.
\item Sensitivity only for suprathreshold.
\item Integration with optogenetics is difficult.
\end{itemize}
Recent development in imaging techniques


2. Faster laser scanning device:
   - Resonant Galvo: 30 – 120 frames/s
   - AOD (Acousto-Optic Deflector): 1000 frames/s.
The improved GECl: GCaMP6

The improved GECI: GCaMP6

Chen T et al., *Nature* 2013
Single spike detection with GCaMP6

Chen T et al., *Nature* 2013
The DIY Microscope

MIMMS (Modular In vivo Multiphoton Microscopy System), Design from HHMI JFRC
Voltage Imaging: Genetically Encoded Voltage Indicators

- **ASAP1**, St-Pierre, … Lin, *Nat Neuroscience* 2014
Polarized mutation that has been shown to shift the voltage response of HEK293A human embryonic kidney cells (GgVSD) that were well expressed at the plasma membrane and showed a fluorescence decrease in response to membrane hyperpolarizations, respectively. In HEK293A cells, ASAP1 produced large responses (∆F/F) or with a 100-Hz fourth order low-pass Butterworth filter (F). (P = 10 cells). Error bars (s.e.m.) are too small (0.2–1.2% in absolute value) to be detected spontaneous (Figure 3A) and evoked action potentials and postsynaptic potentials in neurons. When expressed in neurons, the signal-to-noise ratio of individual action potentials and postsynaptic potentials in excitable cells increased with ASAP1 (left) and ArcLight Q239 (right). The fact that aspartic acid, an acidic amino acid which is modified by the A227D mutation, did not affect membrane expression of ASAP1, but lowered the fluorescence response to hyperpolarizing depolarizations in the form of simulated excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs) of 5–20-mV amplitude, comparing favorably with ArcLight Q239 (∆F/F) in neurons (Figure S4D). We are presently attempting to use lentiviral vectors also for the in vivo expression of ASAP1 constructs in vivo. ASAP1 produced a fluorescence response of 0.6% of the full activation and deactivation ∆F/F or with a 100-Hz fourth order low-pass Butterworth filter (F) or with a 100-Hz fourth order low-pass Butterworth filter (F).


**ASAP1**, St-Pierre, … Lin, Nat Neuroscience 2014
Voltage Imaging: Genetically Encoded Voltage Indicators

QuasAr

Optopatch: QuasAr + CheRiff

QuasAr: Archaerhodopsin-based GEVI

CheRiff: A blue-shifted channelrhodopsin

Hochbaum & Zhao, Nat Methods, 2014.
Voltage Imaging: Genetically Encoded Voltage Indicators

Advantages:

• Fast
• Voltage sensing (supra- and subthreshold)
• Genetically targetable

Limitations:

• Single photon illumination: limited application in highly scattering tissue (in vivo).
• Requiring high speed camera: limiting spatial resolution.

2P imaging is still the best choice for in vivo applications.
Any Questions are Welcome!