



**HAL**  
open science

## Two-photon probes for in vivo multicolor microscopy of the structure and signals of brain cells

Clément Ricard, Erica D Arroyo, Cynthia X He, Carlos Portera-Cailliau, Gabriel Lepousez, Marco Canepari, Daniel Fiole

► **To cite this version:**

Clément Ricard, Erica D Arroyo, Cynthia X He, Carlos Portera-Cailliau, Gabriel Lepousez, et al.. Two-photon probes for in vivo multicolor microscopy of the structure and signals of brain cells. *Brain Structure and Function*, 2018, 223 (7), pp.3011 - 3043. 10.1007/s00429-018-1678-1 . hal-01872748

**HAL Id: hal-01872748**

**<https://hal.science/hal-01872748v1>**

Submitted on 12 Sep 2018

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution 4.0 International License

1 **Two-photon probes for *in vivo* multicolor**  
2 **microscopy of the structure and signals of brain**  
3 **cells.**

4 Clément Ricard<sup>1,2,3</sup>, Erica D. Arroyo<sup>4</sup>, Cynthia X. He<sup>4</sup>, Carlos Portera-Cailliau<sup>4,5</sup>,  
5 Gabriel Lepousez<sup>6</sup>, Marco Canepari<sup>7,8,9</sup>, Daniel Fiole<sup>10,11,\*</sup>

6

7 1) Brain Physiology Laboratory, CNRS UMR 8118, Paris, F-75006, France.

8 2) Faculté de Sciences Fondamentales et Biomédicales, Université Paris Descartes, PRES  
9 Sorbonne Paris Cité, Paris, F-75006, France.

10 3) Fédération de Recherche en Neurosciences FR 3636, Paris, F-75006, France.

11 4) Department of Neurology, David Geffen School of Medicine, University of California, Los  
12 Angeles, United States.

13 5) Department of Neurobiology, David Geffen School of Medicine, University of California, Los  
14 Angeles, United States.

15 6) Unité Perception et Mémoire, Département de Neurosciences, Institut Pasteur, 25 rue du  
16 Docteur Roux, F-75724 Paris Cedex 15, France.

17 7) Laboratory for Interdisciplinary Physics, UMR 5588 CNRS and Université Grenoble Alpes,  
18 F-38402 Saint Martin d'Hères, France.

19 8) Laboratories of Excellence, Ion Channel Science and Therapeutics, France.

20 9) Institut National de la Santé et Recherche Médicale (INSERM), France.

21 10) Unité Biothérapies anti-Infectieuses et Immunité, Département des Maladies Infectieuses,  
22 Institut de Recherche Biomédicale des Armées, BP 73, F-91223 Brétigny-sur-Orge cedex,  
23 France.

24 11) Human Histopathology and Animal Models, Infection and Epidemiology department, Institut  
25 Pasteur, 28 rue du docteur Roux, F-75725 Paris Cedex 15, France.

26 \* corresponding author: [daniel.fiole@gmail.com](mailto:daniel.fiole@gmail.com).

27

28 Current address of DF: CREATIS, CNRS UMR 5220; Inserm U1044; Université de Lyon;  
29 Université Lyon 1; INSA-Lyon, 69621 Villeurbanne, France, and: European Synchrotron  
30 Radiation Facility, X-ray Imaging Group, 38043 Grenoble cedex, France. Current email  
31 address: [fiole@esrf.fr](mailto:fiole@esrf.fr).

32

33 **ORCID iD:** CH 0000-0003-1236-3151; CPC 0000-0001-5735-6380; GL 0000-0002-9390-  
34 7749; DF 0000-0002-1072-6935.

35

36 **[Keywords]**

37 Two-photon cross section, calcium imaging, functional imaging, electroporation, intravital,  
38 multicolor microscopy.

39

## 40 **Abstract**

41 Imaging the brain of living laboratory animals at a microscopic scale can be achieved by two-  
42 photon microscopy thanks to the high penetrability and low phototoxicity of the excitation  
43 wavelengths used. However, knowledge of the two-photon spectral properties of the myriad  
44 fluorescent probes is generally scarce and, for many, non-existent. Additionally, the use of  
45 different measurement units in published reports further hinders the design of a  
46 comprehensive imaging experiment.

47 In this review, we compile and homogenize the two-photon spectral properties of more than  
48 280 fluorescent probes. We provide practical data, including the wavelengths for optimal two-  
49 photon excitation, the peak values of two-photon action cross-section or molecular brightness,  
50 and the emission ranges. Beyond the spectroscopic description of these fluorophores, we  
51 discuss their binding to biological targets. This specificity allows *in vivo* imaging of cells,  
52 their processes, and even organelles and other subcellular structures in the brain. In addition  
53 to probes that monitor endogenous cell metabolism, studies of healthy and diseased brain  
54 benefit from the specific binding of certain probes to pathology-specific features, ranging  
55 from amyloid- $\beta$  plaques to the autofluorescence of certain antibiotics. A special focus is  
56 placed on functional *in vivo* imaging using two-photon probes that sense specific ions or  
57 membrane potential, and that may be combined with optogenetic actuators. Being closely  
58 linked to their use, we examine the different routes of intravital delivery of these fluorescent  
59 probes according to the target. Finally, we discuss different approaches, strategies, and  
60 prerequisites for two-photon multicolor experiments in the brains of living laboratory animals.

## 61 **Introduction**

62 Two-photon excitation fluorescence microscopy, or two-photon microscopy (TPM), became a  
63 standard and powerful method of investigation in biology due to its excellent penetrability in  
64 living tissues and high spatiotemporal resolution, while inducing low phototoxicity and  
65 photobleaching compared to other optical techniques (Denk et al. 1990; So et al. 2000; Zipfel  
66 et al. 2003b; Helmchen and Denk 2005). In this review, we focus on neuroscience because the  
67 functional and physiological properties of the brain makes it a particularly fecund field for the  
68 application of TPM (Svoboda and Yasuda 2006; Mostany et al. 2015).

69 The sophistication of intravital TPM is currently expanding through three axes: 1. Hardware;  
70 2. Sample preparation and new genetic tools; 3. Fluorescent probes (Crowe and Ellis-Davies  
71 2014). First, as far as hardware there have been improvements and new developments in  
72 excitation sources, scanning, and detection. The popularization of optical parametric  
73 oscillators (OPO), which allow excitation far beyond the traditional Titanium:Sapphire lasers,  
74 helps improve the depth of imaging up to 1.6 mm (Kobat et al. 2011). Deep scattering tissues  
75 can be efficiently observed by wavefront optimization thanks to adaptive optics (Ji et al. 2010;  
76 Wang et al. 2015). New kinds of detectors like GaAsP photomultipliers or hybrid avalanche  
77 photodiodes allow detection of dimmer signals. Also, promising advances in the  
78 miniaturization of two-photon microscopes are enabling simultaneous recordings of neural  
79 circuit activity in freely moving animals (Yu et al. 2015; Zong et al. 2017) while avoiding the  
80 side effects associated with the use of anesthetics (Tran and Gordon 2015; Santisakultarm et  
81 al. 2016).

82 Second, refinements in sample preparation beyond the original glass-covered cranial windows  
83 (Holtmaat et al. 2009) and skull thinning (Yang et al. 2010) now permit chronic live imaging  
84 below the superficial layers of neocortex. For example, a substantial increase in resolution can  
85 be achieved by replacing the single coverslip with a pair of coverslips separated by a thin

86 layer of air, which minimizes the effects of spherical aberration (Estrada et al. 2015). Also,  
87 surgical GRIN lens implantation can be combined with head-mounted miniscopes (Resendez  
88 et al. 2016) or standard two-photon microscopes for imaging of deep brain structures such as  
89 hippocampus (Crowe and Ellis-Davies 2014).

90 *In vivo* TPM imaging also benefits from the use of genetic tools that can target fluorochrome  
91 expression to different brain cells, including promoters for specific cell types (*e.g.*, Thy1 mice  
92 for Layer 5B neurons (Feng et al. 2000), CX3CR1 mice for microglia (Jung et al. 2000)). Cre-  
93 Lox recombination can be fruitfully used to target any fluorescent protein in spatially  
94 restricted patterns, such as different cell types, cortical layers, or brain regions, thanks to the  
95 existence of a number of Cre driver lines. Other genetic strategies (*e.g.*, Cre-ER<sup>T2</sup>) can  
96 temporally restrict expression (Kristianto et al. 2017). Different fluorochromes can be  
97 simultaneously expressed on different cells of the same lineage thus (*e.g.*, the Brainbow  
98 construct (Livet et al. 2007)) or on different cell types by breeding together mice of different  
99 phenotypes (*e.g.*, Thy1-**CFP**/LysM-**GFP**/CD11c-**EYFP** (Fenrich et al. 2013)).

100 Designing new transgenic animal models has been recently made less arduous than previously  
101 by CRISPR/Cas9 (Wang and Qi 2016) and PiggyBac (Woodard and Wilson 2015)  
102 technologies, but remains a time-consuming task that can be overcome by the administration  
103 of exogenous fluorescent probes. Depending on the target and on physical and chemical  
104 properties of the probe, methods of administration can range from intravenous dye injection to  
105 *in utero* electroporation and viral protein transduction.

106 Third, the availability of increasing numbers of two-photon-suitable synthetic dyes or  
107 genetically encoded probes with extended properties has brought new perspectives to the use  
108 of TPM in neuroscience. One of these perspectives consists of two-photon functional  
109 imaging, which makes possible to witness brain cells ‘at work’ thanks to voltage-sensitive

110 dyes, probes that monitor levels of various ions (*e.g.*, Cl<sup>-</sup>, Ca<sup>2+</sup>) (Helmchen 2009), or caged  
111 neurotransmitters that can be photoreleased with TPM (Hess et al. 2014).  
112 Beyond the prolific engineering of new fluorescent proteins that unquestionably and fruitfully  
113 expands the biological applications of TPM (Pak et al. 2015), its routine use is hindered by  
114 the need for a more extensive characterization of spectral properties of existing fluorescent  
115 probes. Recent developments in far-red two-photon excitation make the need for an update of  
116 the spectral characteristics of fluorescent probes even more acute (Herz et al. 2010; Mojzisova  
117 and Vermot 2011). While commercial and academic databases of one-photon absorption  
118 spectra are well documented, the two-photon specifications of most probes are only scarcely  
119 documented, despite a small number of helpful initiatives (Bestvater et al. 2002; Cahalan et al.  
120 2002; Drobizhev et al. 2011; Mütze et al. 2012; Romanelli et al. 2013; Lim and Cho 2013).  
121 In this review, we provide a comprehensive list of two-photon–related spectral and biological  
122 properties of more than 280 fluorescent probes, and discuss different routes of delivery of  
123 such probes into laboratory animals, as well as their actual or prospective relevance to *in vivo*  
124 multicolor TPM of cells, structures, and functions, in the healthy and diseased brain.

125

126

127

## 128 1) Reaching the target

### 129 1.1 Intravenous route

130 Blood vessels transport oxygen and nutrients in the whole organism and can be used to  
131 deliver fluorescent dyes into the tiniest parts of an organ (*cf.* Table 1).

132 Blood vessel labeling requires only the intravenous delivery of a fluorescent dye to efficiently  
133 stain an entire organ's vascular tree, which can then be observed *in vivo* with TPM by a z-

134 stack acquisition (Tozer et al. 2005; Ricard et al. 2013b). Common dyes such as **fluorescein**  
135 or **rhodamine** (*cf.* Table 2) can be used; these are harmless to the animal and cleared from the  
136 circulation after a few hours. As capillaries can be permeable to small molecules, these dyes  
137 are usually conjugated with a large molecular weight dextran that is too large to leak into the  
138 tissue. The molecular weight of the resulting dye can thus be chosen depending on the  
139 permeability of the targeted type of vessel. Neocortical arteries and arterioles can be  
140 specifically labeled using **Alexa Fluor 633** (*cf.* Table 3), which binds to elastin fibres (Shen et  
141 al. 2012). However, when multicolor labeling is required, green or red channels are frequently  
142 chosen through the use of other fluorophores or fluorescent proteins (*e.g.*, **EGFP** or **DsRed**).  
143 Blue-emitting dyes such as **Cascade Blue** have been successfully used in intravital studies  
144 and can efficiently be excited by TPM (Ricard and Debarbieux 2014; Ricard et al. 2016b). It  
145 must be considered, however, that in living tissues the maximum imaging depth is a function  
146 of the wavelengths of both the excitation and fluorescence beam. In the TPM range, high-  
147 energy photons are quickly absorbed by the tissue, resulting in a reduced imaging depth  
148 (König 2000). Such a parameter has to be taken into account in the design of the experiment.  
149 Moreover, fluorescent dyes conjugated to dextrans can be captured by phagocytes resulting in  
150 a long-lasting labeling of these cells (Fenrich et al. 2012; Fiole et al. 2014). Such labeling is  
151 observed in the brain and spinal cord dura mater for several days after a single intravenous  
152 injection and can impede the correct segmentation of blood vessels during image processing.  
153 **Quantum dots** were introduced as an alternative to classic fluorescent dyes. They present low  
154 toxicity, are not engulfed by macrophages, have reduced photobleaching, and can fluoresce in  
155 the red and deep-red range, making them particularly suitable for multicolor imaging  
156 (Mashinchian et al. 2014). It was also demonstrated that excitation of quantum dots using an  
157 optical parametric oscillator can improve imaging depth of vasculature in the brain and the  
158 spinal cord (Kobat et al. 2009, 2011; Ricard et al. 2016a). The fluorescence intermittency of



159 quantum dots (Frantsuzov et al. 2013) should however be taken into consideration especially  
160 for single-particle tracking, unless their nonblinking properties are established (Marchuk et al.  
161 2012; Lane et al. 2014).

162 Intravenous injections of fluorescent dyes have also been used to label other structures.  
163 **Sulforhodamine 101** and **Sulforhodamine B** were reported to leak out of the vasculature and  
164 to stain specifically astrocytes (*cf.* Table 1 and Table 3) without showing adverse reactions on  
165 astrocytic calcium signals or electroencephalographic recording *in vivo* (Appaix et al. 2012;  
166 Vérant et al. 2013). **Sulforhodamine B** was also demonstrated to stain elastic fibers in blood  
167 vessel walls, as well as in muscles after a single intravenous injection (Ricard et al. 2007).

168 Intravenous injection of fluorescent probes can also be used to determine the acidity of tissues  
169 using pH indicators (*cf.* Table 4), or to measure physiological parameters such as cerebral  
170 blood flow (Chaigneau et al. 2003) and blood-brain-barrier permeability (Ricard et al. 2009)  
171 in different pathologies, including vascular occlusion (Schaffer et al. 2006) and brain tumors  
172 (Ricard et al. 2013b, 2016b). Investigating the properties of the blood vessel tree enables the  
173 assessment of side effects of treatments in preclinical trials (Ricard et al. 2013a). For instance,  
174 the blood vessel density of glioblastoma-animals injected with **Rhodamine B** dextran was  
175 recorded over time. Experiments conducted in untreated conditions and after the  
176 administration of bevacizumab, an anti-angiogenic compound, revealed a lack of correlation  
177 between tumor growth and blood vessel density (Ricard et al. 2013b).

178 As an alternative but comparable route to intravenous delivery, intraperitoneal administration  
179 (*cf.* Table 1) of fluorochromes can also be performed to stain structures in the diseased brain  
180 (*cf.* Table 5). For example, in Alzheimer's disease research, amyloid- $\beta$  plaques can be  
181 specifically stained by intraperitoneal administration of **SAD1** (Heo et al. 2013) or **Methoxy-**  
182 **X04** (Klunk et al. 2002), revealing the kinetics of amyloid- $\beta$  plaques growing over months  
183 (Burgold et al. 2011).

184

## 185 1.2 Whole-cell and bulk loading

186 A large population of neurons can be bulk-loaded with a cell membrane-permeable  
187 acetoxymethyl (AM) ester-conjugated indicator form (*e.g.*, **Fura-2-AM** or **Fluo-4-AM**)  
188 (Garaschuk et al. 2006; Brenowitz and Regehr 2014) or dextran-conjugated form, enabling  
189 readout of activity across a network (Yuste et al. 2011; Reeves et al. 2011). Bulk-loading of  
190 ion indicators may be spatially restricted to the soma and most proximal processes of neural  
191 cells. However, Reeves *et al.* have shown that performing morphological reconstructions after  
192 bulk-loading of astrocytes in the CA1 region of the hippocampus from rats can help detecting  
193 calcium transients in distal astrocyte processes (Reeves et al. 2011).

194 Alternatively, chemical ion indicators can be typically delivered into single cells via  
195 micropipettes (enabling electrophysiology) or electroporation (Liu and Haas 2011;  
196 Grienberger and Konnerth 2012). While the chemical indicators have high sensitivity and fast  
197 on-off kinetics allowing for precise temporal resolution of action potentials, they are typically  
198 used in acute experimental preparations (a few hours at most), and they are not amenable for  
199 labeling of specific cell populations (Grienberger and Konnerth 2012).

200

## 201 1.3 Viral transduction

202 In order to allow expression of new genes coding for fluorescent proteins in spatially-  
203 restricted and genetically-defined neurons, researchers have developed a versatile toolbox of  
204 replication-incompetent recombinant viral vectors (*cf.* Table 1) that are devoid of most of  
205 their natural genetic material and loaded with engineered constructs (Nassi et al. 2015). The

206 diversity of available vectors reflects the different specifications of each viral vectors in terms  
207 of:

- 208 1) tropism for cell type, compartment (axonal vs. somato-dendritic), and animal species. This  
209 tropism is directly influenced by the nature of the glycoprotein of the envelope (which defines  
210 the serotype) and the expression of receptors for envelope glycoprotein on targeted cells  
211 leading to vector internalization;
- 212 2) transduction and expression efficiency, speed of expression after infection, and stability for  
213 long term expression;
- 214 3) vector genome size and maximum insert size, ease of manipulating the genome and  
215 producing high-titer solutions, and integration into the host genome;
- 216 4) and immunogenicity, cell toxicity and safety.

217 Here we list the main viral vectors used in the neuroscience field and their general features:

- 218 - retroviruses are integrative vectors with an insert size up to 8-9 kB, providing a stable  
219 long-term expression. They exclusively transduce dividing cells and show moderate  
220 immunogeneticity.
- 221 - lentiviruses are integrative vectors with an insert size up to 8-9 kb, good for stable  
222 long-term expression. They have a large tropism and can transduce most CNS cells  
223 (astrocytes, neurons, and oligodendrocytes) and show moderate immunogeneticity.
- 224 - adeno-associated viruses (AAV) are currently the most commonly used vector for  
225 gene delivery. They are non-integrative vectors with an insert size up to 4-5 kb.  
226 Because of their small size and high titer production, a single injection can infect a  
227 large volume of tissue. They are also favored over other vectors for their mild  
228 immunogenicity and a dominant neuronal tropism. To enlarge their tropism, envelope  
229 proteins have been engineered using directed evolution to target specific cell types  
230 (*e.g.*, oligodendrocytes (Büning et al. 2015; Powell et al. 2016)) or to target specific

231 neuronal compartments (*e.g.*, axonal domain for retrograde labeling of neurons (Tervo  
232 et al. 2016)).

233 - Herpes Simplex Virus (HSV-1) is a non-integrative vector with an insert size up to  
234 100 kb. Although its complex genome is not easy to manipulate, HSV-1s transduce  
235 mainly neurons with a dominant axonal tropism, making them an interesting tool for  
236 retrograde labeling of neurons. Importantly, they show significant immunogenicity  
237 and cell toxicity.

238 - Canine Adenovirus (CAV-2) is a non-integrative vector with a smaller insert size up to  
239 30 kb (Junyent and Kremer 2015). CAV-2s transduce mainly neurons with a dominant  
240 axonal tropism, making them an interesting tool for retrograde labeling of neurons.  
241 The main receptor for the internalization of the virus is the coxsackievirus and  
242 adenovirus receptor (CAR). They also show a significant immunogenicity and cell  
243 toxicity.

244 - Rabies virus (RABV) is a non-integrative vector with an insert size of 4-5 kb. RABVs  
245 are neurotropic and are classically used as replication-conditional pseudotyped viruses  
246 for retrograde tracing of mono-synaptic inputs onto genetically-defined cell  
247 populations (Wickersham et al. 2007). Rabies virus is pseudotyped with the EnvA  
248 glycoprotein to ensure that the virus exclusively infects cells expressing the EnvA  
249 receptor (TVA). The virus also lacks the envelope glycoprotein and expresses the gene  
250 of interest. Complementation of the modified rabies virus with the envelope  
251 glycoprotein in the TVA-expressing cells allows the generation of infectious particles,  
252 which trans-synaptically infect presynaptic neurons. Importantly, rabies virus shows  
253 strong neurotoxicity with longer-term infection (>15 days).

254



## 256 1.4 *In utero* electroporation

257 *In utero* electroporation (IUE) is a technique that enables researchers to express genes of  
258 interest within specific neuronal populations (*cf.* Table 1) by targeting plasmid DNA  
259 constructs directly to the embryonic brain of rodents (Fukuchi-Shimogori and Grove 2001;  
260 Saito and Nakatsuji 2001; Takahashi et al. 2002; Wang and Mei 2013). Although this section  
261 focuses on cerebral cortex, IUE can be used for gene transfer in other brain regions  
262 (Takiguchi-Hayashi et al. 2004; Borrell et al. 2005; Nakahira et al. 2006; Navarro-Quiroga et  
263 al. 2007; Bonnin et al. 2007). For cortical labeling, IUE takes advantage of one of the most  
264 reliable and tightly regulated processes that occurs during brain development: the sequential  
265 inside-out laminar organization of the cortex, whereby neurons in deeper layers are generated  
266 before those in more superficial layers (Angevine and Sidman 1961; Rakic 1974). IUE is  
267 performed at the gestational age that coincides with the generation of pyramidal precursor  
268 cells at the subventricular zone along the lateral ventricles. These newborn neurons eventually  
269 migrate to and incorporate into their appropriate cortical layer (Caviness and Takahashi 1995;  
270 Tabata and Nakajima 2001). For instance, to label layer 2/3 excitatory pyramidal neurons,  
271 IUE is performed at embryonic day (E)15-16 (Saito and Nakatsuji 2001).

272 Although IUE can be used to over-express essentially any protein of interest, it is perhaps  
273 most often used to express fluorescent proteins that make it possible to image neuronal  
274 structure with confocal or two-photon microscopy. Because IUE can provide sparse labeling  
275 in neurons, it is particularly well-suited for high-resolution imaging of the finest detail of  
276 neuronal structure, such as dendritic spines. Another major advantage of IUE over traditional  
277 fluorescence labeling techniques such as transgenic mouse lines, is that it enables researchers  
278 to conduct early postnatal imaging. This is due to the fact that expression of fluorescent  
279 proteins (*e.g.*, **GFP**, **YFP**) in transgenic mouse lines is often driven by promoters that initiate

280 transcription after synaptogenesis has already been completed in neocortex; for example,  
281 cortical expression of **YFP** in Thy1-eYFP-H mice occurs around postnatal day (P) 21 (Feng et  
282 al. 2000; Porrero et al. 2010)). IUE can also achieve potent transduction of fluorescent  
283 proteins through the use of constitutively active promoters such as pCAG or other CMV  
284 variations (Saito and Nakatsuji 2001); this is particularly useful for deep tissue imaging with  
285 *in vivo* TPM. Following IUE, mice can be imaged from perinatal development through  
286 adulthood (Cruz-Martin et al. 2010). Another problem with transgenic lines is that layer  
287 specificity is limited by the fidelity of the promoter itself and specific Cre lines are not yet  
288 available for desired cell types or brain regions, or they may have off target expression. In  
289 contrast, IUE at different embryonic stages can be used to target different cortical layers in  
290 different locations. Furthermore, tailoring DNA plasmid constructs to a particular  
291 experimental design is less expensive and less time-consuming than generating new mouse  
292 lines. Additionally, co-labeling cells with multiple fluorophores using IUE can easily be  
293 achieved by co-injecting multiple plasmids or by using bi-cistronic promoters (*e.g.*, P2A (Kim  
294 et al. 2011)). It is also possible to design plasmids to express opsins or DREADD constructs,  
295 to genetically manipulate subpopulations of neurons both constitutively and conditionally  
296 (Takahashi et al. 2002; Matsuda and Cepko 2004, 2007; Yasuda et al. 2006; Huber et al.  
297 2008; Manent et al. 2009). IUE also affords significant flexibility because the level of  
298 expression can be controlled by varying the voltage delivery, concentration of the plasmid,  
299 and the volume injected. Of note, IUE may not be compatible with the expression of  
300 fluorescent calcium indicators (**GCaMP6**) as anecdotal reports suggest that few if any  
301 neurons survive postnatally, presumably due to some toxicity from calcium buffering.  
302 In conclusion, IUE is a powerful method for the expression of proteins in the early postnatal  
303 brain and into adulthood, especially for layer-specific cortical neurons.

304

## 305 2) Interrogating neural signals

### 306 2.1 Calcium imaging

307 The ability to measure changes in ion levels in living tissues with fluorescent microscopy  
308 offers many possibilities for scientific investigations (*cf.* Table 6). Across cell types and  
309 within multiple intracellular compartments, calcium ions ( $\text{Ca}^{2+}$ ) play a variety of important  
310 roles, including cell cycle regulation, gene transcription modulation, intracellular signaling,  
311 muscle contraction and neurotransmission (Grienberger and Konnerth 2012). Action  
312 potentials in neurons result in massive influxes of  $\text{Ca}^{2+}$  through voltage-gated channels, as  
313 well as the release of  $\text{Ca}^{2+}$  from intracellular stores (Kandel et al. 2000), and fluctuations in  
314 free  $\text{Ca}^{2+}$  in the presynaptic and postsynaptic compartments contribute to activity-dependent  
315 plasticity (Grienberger and Konnerth 2012). Because changes in the level of intracellular  $\text{Ca}^{2+}$   
316 are a robust indicator of action potential firing in neurons, fluorescent  $\text{Ca}^{2+}$  indicators have  
317 become powerful tools for recording neural activity with excellent spatial and temporal  
318 resolution. Additionally, different types of  $\text{Ca}^{2+}$  signaling events have been studied in  
319 astrocytes (Srinivasan et al. 2015) and in cardiomyocytes (Herron et al. 2012).

320 In general,  $\text{Ca}^{2+}$  imaging relies on a fluorescent sensor compound that is introduced into  
321 neurons (or other cells) and that, when bound to  $\text{Ca}^{2+}$ , changes its fluorescent properties. The  
322 two primary classes of  $\text{Ca}^{2+}$  indicators are the synthetic chemical indicators and the  
323 genetically encoded indicators. The chemical calcium indicators (*e.g.*, **Fura-2**, **Indo-1**, **Fluo-**  
324 **4**, **Oregon Green BAPTA-1**) were pioneered by Roger Tsien's group and utilize a synthetic  
325  $\text{Ca}^{2+}$  chelator combined with a fluorophore (Grynkiewicz et al. 1985; Tsien et al. 1985; Brain  
326 and Bennett 1997; Gee et al. 2000). When  $\text{Ca}^{2+}$  binds to the chelator site, the molecule  
327 undergoes a conformational change that alters the spectrum of emitted fluorescence



328 (Grienberger and Konnerth 2012). **Fura-2** is excited by ultraviolet wavelengths, produces  
329 peak fluorescence at 505-520 nm, and has relatively fast kinetics (Tsien et al. 1985). Under  
330 two-photon excitation, **Fura-2** fluorescence decreases as  $[Ca^{2+}]$  increases, producing  
331 decreases in fluorescence intensity during neuronal activity. In contrast, the fluorescence  
332 emitted by **Fluo-4** and **Oregon Green BAPTA-1** increases above its baseline as  $[Ca^{2+}]$   
333 increases during action potential firing.

334 The genetically encoded  $Ca^{2+}$  indicators (GECIs) use a  $Ca^{2+}$  binding protein, such as  
335 calmodulin or troponin, instead of a chelator like BAPTA. Since the discovery of the green  
336 fluorescent protein, scientists have generated an array of sensors combining **GFP** variants  
337 with  $Ca^{2+}$ -binding proteins. The GECI subcategory of cameleons (Miyawaki et al. 1997), is  
338 perhaps the most popular amongst GECIs, but other varieties exist based on troponin-C as the  
339  $Ca^{2+}$ -binding protein (Mank et al. 2008). Yellow Cameleon (Nagai et al. 2004) utilizes Förster  
340 resonance energy transfer (FRET) between two different fluorescent proteins, linked by  
341 calmodulin and calmodulin binding peptide M13. Upon calmodulin binding to  $Ca^{2+}$ , the  
342 conformational change brings the two fluorophores – one **ECFP** and one **Venus-YFP** – close  
343 enough to result in activation of the yellow, resulting in a measurable change in the  
344 cyan:yellow fluorescence ratio (Nagai et al. 2004; Grienberger et al. 2014).

345 For *in vivo* two-photon  $Ca^{2+}$  imaging, the most frequently used GECI is the cameleon-based  
346 **GCaMP** variety, which utilizes a single circularly permuted green fluorophore (**GFP**)  
347 attached to calmodulin and the M13 peptide, and is maintained in a low fluorescence state  
348 when  $Ca^{2+}$  is not bound (Nakai et al. 2001).  $Ca^{2+}$  binding to calmodulin causes a  
349 conformational shift that changes the solvent exposure of the **GFP** and allows a fluorescence  
350 increase (Chen et al. 2013). Earlier versions of **GCaMP** indicators had relatively low slow  
351 on-off kinetics and signal-to-noise ratio, which could however be improved by 3D Block-  
352 Matching filtering (Danielyan et al. 2014). More recently, the development of the

353 “ultrasensitive” **GCaMP6** has improved neuronal event detection capability to single-spike  
354 resolution, though the off-kinetics remain somewhat slow (Chen et al. 2013). Typical  
355 expression methods for GECIs include viral transduction (Chen et al. 2013), with the  
356 associated limitation of eventual cytotoxicity caused by long-term calcium sequestration; and  
357 transgenic mice expressing **GCaMP** (Zariwala et al. 2012; Chen et al. 2012; Dana et al.  
358 2014). Finally, it is important to note that continual improvements have also been made in  
359 red-shifted GECIs (Looger and Griesbeck 2011), with the most recent iterations being  
360 **jRCaMP1a**, **jRCaMP1b**, and **jRGECO1a** (Dana et al. 2016). Further developments in these  
361 latter GECIs will allow neuroscientists to record from even deeper brain structures, due to the  
362 reduced scattering of longer-wavelength excitation light.

363

## 364 2.2 Sodium imaging

365 Another important ion that can be measured intracellularly is  $\text{Na}^+$ . In contrast to  $\text{Ca}^{2+}$   
366 indicators,  $\text{Na}^+$  indicators are designed to measure  $\text{Na}^+$  concentration in millimolar ranges and  
367 therefore these dyes have significantly lower affinity. A commonly used indicator is **SBFI**  
368 that is excitable in the UV range and has ratiometric properties similar to **Fura** indicators.  
369 This indicator has been used to measure  $\text{Na}^+$  in neurons (Myoga et al. 2009) and astrocytes  
370 (Langer and Rose 2009). An alternative indicator excitable with blue light is **Sodium Green**  
371 that was also used to measure  $\text{Na}^+$  in neurons (Senatorov et al. 2000). More recently, a greater  
372 sensitivity for fast  $\text{Na}^+$  changes in neuronal axons was found for the green excitable indicator  
373 **ANG-2** (Miyazaki and Ross 2015).

374

## 375 2.3 Voltage-sensitive dyes

376 One problem with  $\text{Ca}^{2+}$  imaging is that the kinetics of the dyes, on the order of hundreds of  
377 milliseconds or seconds, are orders of magnitude slower than the duration of typical action  
378 potentials. As a result, one cannot record neural activity with precise temporal resolution,  
379 which is critical for phenomena like spike timing dependent plasticity. Ideally, one would  
380 want to record changes in membrane potential ( $V_m$ ), using either organic (*cf.* Table 6) or  
381 genetically encoded voltage sensors (GEVS), which have exquisite temporal resolution. *In*  
382 *vivo* voltage-sensitive dye (VSD) imaging from large cell populations in the anesthetized  
383 mammalian brain was developed in the nineties (Shoham et al. 1999; Petersen et al. 2003;  
384 Grinvald and Hildesheim 2004). This approach is sometimes coupled with intracortical  
385 microstimulation and electrode recordings, and requires either injection or topical application  
386 (Murphy et al. 2008) of oxonol VSDs, such as **RH-1692**. These have been designed to absorb  
387 light in the red region and are therefore outside the absorption band of haemoglobin (which  
388 causes pulsation and hemodynamic noise in brain recordings). More recently, styryl VSDs  
389 with similar spectral properties were developed (Zhou et al. 2007). The techniques of *in vivo*  
390 VSD imaging from large cell populations, using oxonol dyes, further progressed until  
391 enabling recordings from the barrel cortex of awake head-fixed mice (Poulet and Petersen  
392 2008) or from freely moving animals (Ferezou et al. 2006).

393 Among the other *in vivo* applications of VSD imaging, it is important to mention the studies  
394 on embryonic developing nervous systems (Kamino et al. 1989). For this application,  
395 absorption VSDs such as NK2429 (Fujii et al. 1981) are typically used, since the high  
396 translucency of embryonic tissue allows high-sensitivity absorption measurements (Momose-  
397 Sato et al. 2001). To achieve *in vivo*  $V_m$  imaging with cellular or subcellular resolution, two-  
398 photon excitation can be used. To this purpose, the two-photon cross-sections of several styryl  
399 VSDs were measured (Fisher et al. 2005). Action potentials from mammalian nerve terminals  
400 were then recorded in an *ex vivo* preparation, the neurohypophysis (*pars nervosa*), using the

401 VSD **di-3-ANEPPDHQ** (Fisher et al. 2008). More recently, some novel fluorinated VSDs  
402 (Yan et al. 2012) that appear to be more photostable when excited by two photon were  
403 developed, and have been used to resolve action potentials from dendritic spines in brain  
404 slices (Acker et al. 2011).

405 In many cases, *in vivo* two-photon  $V_m$  imaging requires the topical application of two-photon  
406 suitable VSD (*cf.* Table 1) after either craniotomy (Murphy et al. 2008). This challenging  
407 approach is highly rewarding as it allows  $V_m$  imaging during wakefulness after topical  
408 administration of **ANNINE-6** (Kuhn et al. 2008), opening the door for simultaneous  $V_m$   
409 imaging and behavioral tests.

410 In summary, *in vivo*  $V_m$  imaging using organic indicators has several practical limitations  
411 associated with dye loading, access to small structures, and stability of recordings. In terms of  
412 future prospective, most of these limitations can be overcome by replacing organic indicators  
413 with GEVS that can be expressed *in vivo* by viral transduction (Marshall et al. 2016). The first  
414 generation of GEVS was developed by mutating the *Drosophila* Shaker potassium channel  
415 and fusing it to a **GFP** protein (Siegel and Isacoff 1997). While good optical responses to  $V_m$   
416 changes could be obtained by expressing the sensor in oocytes, the protein could not be  
417 expressed in the plasma membrane of mammalian neurons and therefore a second generation  
418 of GEVS was developed (Dimitrov et al. 2007). These GEVSs, now expressed in neuronal  
419 outer membranes, were based on the voltage-sensitive phosphatase from the sea squirt, *Ciona*  
420 *intestinalis*, fused to a **CFP** and **YFP** FRET pair (Lundby et al. 2008). From these pioneering  
421 works, new GEVS based either on single FRET pairs (Jin et al. 2012) or on **GFP** probes (St-  
422 Pierre et al. 2014) have been more recently developed with faster responses and enhanced  
423 sensitivity, opening the gate to future explorations of activity in the living brain.

424

425

426

427

## 428           2.4 Optogenetics actuators

429   Optogenetic technology has become an essential tool to study the structure and the function of  
430   the neural circuits underlying behavior and cognition. Using photosensitive microbial opsin  
431   genes expressed into genetically defined neurons, optogenetics provides millisecond-precision  
432   control —activation or inhibition — of defined circuit elements in intact organisms with light  
433   (reviewed by Deisseroth (Deisseroth 2015)). Recent developments have provided a large  
434   diversity of excitatory and inhibitory opsins that are sensitive to different wavelengths. For  
435   instance, red-light-activated opsins (such as C1V1, ChRimson) allow simultaneous activation  
436   of neurons and imaging of neuronal activity with the green genetic probe **GCaMP**.  
437   Optogenetic tools have been upgraded to achieve two-photon manipulation of neuronal  
438   populations at the single-cell resolution. The red-light-activated C1V1 opsin allows the robust  
439   generation of precise and fast spike trains using 1,040-nm light and standard raster-scanning  
440   light delivery (Prakash et al. 2012). This tool can also be used in combination with two-  
441   photon imaging of **GCaMP** probe using 920-nm light (Rickgauer et al. 2014; Packer et al.  
442   2015). Inhibitory opsins such as eNpHR3.0 have also proven to be efficient for neuronal  
443   inhibition using 1,040 nm light (Prakash et al. 2012). New illumination methods now exist to  
444   improve opsin activation, such as spiral scanning, temporal focusing and holography-based  
445   patterned light illumination (Papagiakoumou et al. 2013). Thus, combining optogenetics with  
446   imaging technologies opens new avenues to all-optical interrogation of neuronal circuits with  
447   closed-loop manipulation of neurons in real time (Emiliani et al. 2015).

448

449

## 2.5 Spectral properties of endogenous molecules

450 Utilizing the intrinsic spectral properties of the target tissue, when possible, places the  
451 experimenter in a very favorable situation by avoiding the complicated, invasive, and/or  
452 possibly bias-inducing administration and binding of exogenous fluorescent probes.

453 Many, if not all endogenous molecules display a certain capacity of two-photon excitation,  
454 though this excitation is generally rather inefficient. What appears in most cases to produce an  
455 annoying background noise does carry some meaningful information. The two-photon action  
456 cross-section spectra of several intrinsic molecules (among which **NADH**, **riboflavin**, **folic**  
457 **acid**, **cholecalciferol**, **pyridoxine**, and **retinol**) has been assessed in the early 2000s by the  
458 laboratory of Watt W. Webb at Cornell (Zipfel et al. 2003a), facilitating the two-photon–  
459 based analysis of metabolism (*cf.* Table 7). Investigating cell dysfunction in a number of  
460 pathologies is made possible through the measurement of the fluorescence decay using  
461 fluorescence lifetime imaging (FLIM) of metabolites. For instance, **NADH** FLIM-  
462 experiments allowed a better characterization of tumor-associated microglia phenotypes  
463 (Bayerl et al. 2016), unveiled the role of astrocytes in chronic neuroinflammation-related  
464 neuronal death (Mossakowski et al. 2015; Radbruch et al. 2016), and provided a label-free  
465 and non-invasive mean to identify neuron- or glial- biased progenitors (Stringari et al. 2012).  
466 Moreover, simultaneous FLIM-based detection of several metabolites like **NADH** and **FAD**  
467 with SHG can be achieved through wavelength mixing (Stringari et al. 2017) (for more  
468 details, see section *Designing a microscopy setup for intravital multicolor TPM*).

469 Although not endogenous, it is worth mentioning the very few instances of therapeutic agents  
470 whose two-photon spectral characteristics are known: the fluoroquinolone antibiotics  
471 **gatifloxacin** and **moxifloxacin** (Lee et al. 2016) (*cf.* Table 5), and **quinacrine** (Bestvater et  
472 al. 2002) which stains nucleic acids (*cf.* Table 8) while having several therapeutic indications  
473 (Wallace 1989; Eriksson et al. 2015; Lippes 2015).

## 474 3) Combining fluorescent probes for multicolor TPM

### 475 3.1 Designing a multicolor animal model

476 The first point to consider when designing an intravital multicolor TPM experiment is the co-  
477 labeling of multiple targets in the biological sample. Such labeling can result from crossing  
478 transgenic mice that express fluorescent proteins in different cells or structures of interest. For  
479 instance, triple transgenic fluorescent mice were described and successfully involved in  
480 intravital multicolor TPM experiments (Fenrich et al. 2013; Ricard and Debarbieux 2014).  
481 However, producing such animals requires many crossings that considerably delay their use  
482 and increase the cost of the experiment. Another approach to multicolor imaging is the  
483 Brainbow transgenic mouse, which takes advantage of Cre/lox recombination to stochastically  
484 express different fluorescent proteins in a cell population. As a consequence, each cell  
485 randomly express a combination of fluorescent proteins and thus can be followed individually  
486 (Livet et al. 2007; Weissman and Pan 2015). Different probes can be used to highlight  
487 structures and/or functions (*e.g.*, VSD or calcium-sensitive dyes) (Akemann et al. 2013;  
488 Shigetomi et al. 2013, 2016; Xu et al. 2017).

489 Taking advantage of the intrinsic properties of endogenous molecules is an elegant way to  
490 investigate biological tissues without the drawbacks associated with the administration,  
491 binding and possibly metabolize of exogenous molecules. Second-harmonic generation  
492 (SHG) can be used to visualize not only the meninges via type-I or -III collagen imaging  
493 (Williams et al. 2005; Ricard et al. 2007; Keikhosravi et al. 2014; Ricard and Debarbieux  
494 2014), but also membrane potential (Rama et al. 2010; Loew and Lewis 2015). Third-  
495 harmonic generation (THG) highlights water-lipids and water-proteins interfaces (Débarre et  
496 al. 2006; Weigelin et al. 2016) and was successfully tested *in vivo* to image neurons, white-  
497 matter structures, and blood vessels simultaneously (Witte et al. 2011). Both SHG and THG



498 require no staining at all and are observed at half or one-third of the excitation wavelength  
499 respectively. Beside the respective specificity of SHG and THG, they have been fruitfully  
500 used to record morphological landmarks and help data registration during longitudinal *in vivo*  
501 imaging of neural zebrafish stem cells (Dray et al. 2015). Autofluorescence does arise from  
502 molecules and structures under specific excitation wavelengths (Zipfel et al. 2003a; Ricard et  
503 al. 2012). At best autofluorescence may carry relevant information, and at worst, being aware  
504 of it may help achieve higher contrast between the background and structures of interest by  
505 choosing fluorophores accordingly. It must be noted that autofluorescence signals are not very  
506 bright in intravital conditions (*cf.* Table 7) and were mainly described in explants or tissue  
507 slices. Due to the low intensity of endogenous signals, one could be tempted to increase the  
508 laser power. However, this usually causes artifacts to appear, whose monitoring can help in  
509 evaluating photodamage induced during the experiment (Galli et al. 2014).

510 The preparation of a biological sample for intravital multicolor TPM often requires combining  
511 different approaches to label a maximal number of structures of interest involved in a  
512 complex process. For example, using Thy1-**CFP**/LysM-**GFP**/CD11c-**EYFP** triple transgenic  
513 mice intravenously injected with **Rhodamine B** dextran or Quantum-dots 655, Fenrich *et al.*  
514 have revealed a differential spatiotemporal recruitment of myelomonocytic cells after a spinal  
515 cord injury (Fenrich et al. 2013). Recently, Ricard *et al.* have described the combination of a  
516 LysM-**EGFP**/CD11c-**EYFP** mouse implanted with **DsRed**-expressing glioblastoma cells and  
517 intravenously injected with **Cascade Blue** dextran in order to study the recruitment of  
518 immune cells during glioblastoma progression (Ricard et al. 2016b).

519 The development of the co-staining protocol of a biological sample to be used for intravital  
520 multicolor TPM must follow some guidelines:

521 1) Spectral overlap of the emission spectra of all of the utilized fluorophores must be minimal,

522 2) Utilized fluorophores must not bias physiological and biological properties of the sample  
523 nor the pathological condition that is observed,

524 3) As multiple-fluorophore-expressing animals are difficult to produce, the experimental  
525 design should take into account the risk of a downsized animal cohort.

526

527

## 528 3.2 Designing a microscopy setup for intravital multicolor TPM

529 The next point to consider, when multicolor TPM is required for an experiment, is the  
530 microscopy setup (*cf.* Figure 1). Excitation spectra of the fluorophores are the first elements to  
531 take into account. The majority of blue-, green- and red-emitting fluorophores can be excited  
532 with a Ti:Sapphire femtosecond laser, as its emission wavelength is generally tunable in  
533 between 700 and 1,040 nm. When possible, it is advisable to use a single excitation  
534 wavelength and tune precisely the gain of detectors associated with each fluorophore. The  
535 laser power should be kept as low as possible and the experiment duration as short as  
536 possible, to limit photobleaching and phototoxicity. This is essential for multicolor timelapse  
537 experiments (Fenrich et al. 2013; Ricard et al. 2016b), in which the same region is scanned  
538 over and over, at the risk of exposing the tissue to an energy that would threaten its health  
539 and/or integrity. When different excitation wavelengths are required (for example, when both  
540 **Cascade Blue** with  $\lambda_{\text{ex}} = 800$  nm and **eGFP** with  $\lambda_{\text{ex}} = 940$  nm are present in the same  
541 biological sample), a sequential acquisition or the simultaneous use of two Ti:Sapphire  
542 femtosecond lasers is required. Continuous sequential acquisition (for example, for calcium  
543 imaging recordings) increases the total acquisition time of the images, which is of concern as  
544 far as preventing photodamage and prolonged exposure of the animal to anesthetics (if  
545 applicable), or depending on the characteristic time of the studied phenomenon.

546 Deep-red fluorophores require higher excitation wavelengths that cannot be delivered by  
547 Ti:Sapphire femtosecond lasers alone. Typically, they are excited by Ytterbium lasers (1,040  
548 nm) but the addition of an OPO (Herz et al. 2010) pumped by a Ti:Sapphire femtosecond  
549 laser can enable excitation wavelengths ranging from 1,050 to 1,300 nm when pumped at 800  
550 nm. Simultaneous utilization of both a Ti:Sapphire femtosecond laser and an OPO is  
551 achievable. Interestingly, it enables the simultaneous two-photon excitation of three  
552 fluorophores with distinct absorption spectra insofar as the pulses of the laser and OPO are

553 synchronized. The excitation of the three fluorophores is done by the femtosecond laser, the  
554 OPO and by their spatiotemporal overlap that produces a third two-photon excitation. With  
555 such a wavelength mixing, **CFP**, **YFP** and **tdTomato** – having distinct two-photon absorption  
556 spectra – can be simultaneously excited. Biological phenomena such as cell movements in  
557 embryonic tissues were thereby monitored over time using Brainbow constructs (Mahou et al.  
558 2012). Endogenous metabolites such as **NADH** and **FAD** can also be detected by FLIM,  
559 simultaneously with the SHG of surrounding tissues, using a wavelength mixing approach  
560 (Stringari et al. 2017). In this case, two-photon excitations are produced by the  
561 synchronization of two excitation beams at 760 nm (**NADH** and **FAD**) and 1041 nm that  
562 creates a third (virtual) wavelength at 879 nm (**FAD**) (*cf.* Figure 2).

563 More recently, the efficient detection of up to 7 tissue compartments (five fluorophores, SHG  
564 from collagen, and autofluorescence) has been reported using wavelength mixing combined  
565 with both a broad set of fluorophores (*cf.* Figure 3) and a dedicated spectral unmixing  
566 algorithm (Rakhymzhan et al. 2017) (for more details, see section *Image processing*).

567 The next element to consider is the fluorescence collection. Fluorescence photons are nearly  
568 always collected in non-descanned mode in intravital TPM, *i.e.*, without passing back through  
569 the scanning mirrors. They are instead reflected on a dichroic mirror situated between the  
570 objective and scanning mirrors, and are directed towards photomultiplier tubes (PMT), also  
571 known as non-descanned detectors (NDDs). In multicolor TPM, a number of NDDs is used.  
572 Fluorescence photons are discriminated according to their wavelengths using dichroic mirrors  
573 and filters. With such a setup, fluorescence arising from different fluorophores can be  
574 simultaneously collected on distinct detectors. Fluorescence intensity varies between the  
575 different used fluorophores according to parameters such as their quantity, their two-photon  
576 absorption cross-section ( $\sigma_2$ ) and quantum yield ( $\phi$ ), the product of which defining the two-  
577 photon action cross-section ( $\sigma_2\phi$ ) listed in Tables 2-8. The emission wavelength of a

578 fluorophore is another parameter influencing the collected intensity when put into perspective  
579 with the NDD response curve, high-energy blue photons being more absorbed than low-  
580 energy red photons in biological tissues (König 2000). When a single excitation is used, the  
581 laser intensity delivered to each of the fluorophores at the same observation depth will be  
582 identical. NDD gain should be adjusted accordingly for each of the fluorophores in order to  
583 enhance the signal over noise ratio, while avoiding saturating the detectors. When one expects  
584 a specific fluorescence signal to be low, more efficient PMTs such as Gallium Arsenide  
585 Phosphide (GaAsP) detectors should be considered (Becker et al. 2011).

586 Multicolor intravital TPM can benefit from the use of spectral chips, composed of an array of  
587 PMTs and a prism or diffraction grating to separate the fluorescence photons (Im et al. 2010;  
588 Shi et al. 2012, 2015; Zimmermann et al. 2014). Spectral segmentation of the wavelengths  
589 arising for the different fluorophores will be thereby both more accurate and easier to achieve.  
590 Although such chips are already available on commercial confocal microscopes, they are  
591 positioned on the descanned path that is less suitable for intravital applications. Spectral chips  
592 in a non-descanned mode are required, and recent advances in non-descanned collection of  
593 fluorescence photons by optical fibers may help develop this approach (Ducros et al. 2011).

594

595

### 596 3.3 Image processing

597 Multicolor intravital timelapse TPM generates large amounts of data. The aim of the post-  
598 processing steps is to extract quantitative data and relevant characterization of the  
599 phenomenon of interest from multicolor acquisition.

600 When required, the first step is to perform spectral unmixing (Zimmermann et al. 2002; Neher  
601 and Neher 2004; Ducros et al. 2009). Even though the emission spectra of all of the  
602 fluorophores used in the experiment do not peak at the same wavelengths, there is generally a  
603 certain overlap of the spectra. As a consequence, the signal collected on one NDD contains a  
604 major contribution from one fluorophore and minor contributions from the others. It is  
605 possible to overcome this problem and to clean the image to retain only the contribution of the  
606 major fluorophore. To perform this spectral unmixing (*a.k.a.*, spectral deconvolution), the  
607 contribution of each of the fluorophores used in the experiment in each of the NDD must be  
608 measured. Then, using dedicated algorithms based on maximum-likelihood unmixing (Davis  
609 and Shen 2007), each image from each NDD can be spectrally unmixed and then analyzed  
610 (Thaler and Vogel 2006; Brenner et al. 2013; Zimmermann et al. 2014). As an example of the  
611 crucial importance of spectral unmixing, Ducros *et al.* have used it to detect small spectral  
612 variations of odor-evoked FRET transients up to 250  $\mu\text{m}$  in the olfactory bulb of living mice  
613 (Ducros et al. 2009).

614 In another instance of multicolor intravital TPM experiment, Ricard *et al.* have recently  
615 proposed the 6-color intravital TPM of brain tumors (Ricard and Debarbieux 2014). They  
616 designed a triple transgenic Connexin43-**CFP**/Thy1-**GFP**/CD11c-**YFP** mouse model enabling  
617 the simultaneous observation of astrocytes, neurons, and microglia/dendritic cells,  
618 respectively. Mice were grafted under a chronic cranial window with glioblastoma cells  
619 expressing **DsRed**. Blood vessels were highlighted by an intravenous delivery of **Cascade**  
620 **Blue** dextran. Meninges were observed using SHG. Images were acquired on a microscope

621 equipped with 5 NDDs under a sequential excitation at 800 and 940 nm. This sequential  
622 acquisition allows a discrimination of **CFP** and **Cascade Blue** signals as their two-photon  
623 excitation spectra are different, even though their emission spectra strongly overlap. After  
624 spectral unmixing, analysis revealed in particular significant alterations of cell motility in the  
625 peritumoral area.

626 The usefulness of spectral unmixing algorithms has become even clearer with the successful  
627 attempt to simultaneously detect up to 7 tissue compartments using two two-photon excitation  
628 sources and 6 NDDs (Rakhymzhan et al. 2017). In order to do this, the authors of this work  
629 developed a dedicated pixel-based algorithm based both on similarity measurements between  
630 overlapping fluorophores and on the spectral fingerprints of the individual fluorophores. As a  
631 result, the ‘SIMI’ algorithm enables the separation of more fluorophores than available  
632 channels (*cf.* Figure 4). This protocol has been used in murine lymph nodes to simultaneously  
633 detect *in vivo* lymphocytes labelled with **Hoechst**, **CFP**, hrGFP, **YFP**, or **DsRed**, plus SHG  
634 and autofluorescence from macrophages.

## 635 **Conclusion**

636 Intravital TPM is a powerful tool to visualize biological phenomena with a subcellular  
637 resolution in a physiological environment. Multicolor TPM experiments make the most of the  
638 presence of all the actors – known or yet unknown – exhaustively involved in the complex  
639 processes that characterize brain functions. The design of intravital multicolor TPM  
640 experiments is, however, hindered at different stages following the identification of the  
641 biological targets of interest: choice of suitable fluorophores, choice of appropriate routes of  
642 delivery of these fluorophores, and design of the setup allowing simultaneous detection of  
643 various fluorophores.

644 This review was intended to reduce these hurdles by providing a comprehensive list of two-  
645 photon-suitable probes along with their spectral properties and biological specificities (*cf.*  
646 Tables 2 to 8), a description of different routes of *in vivo* delivery of these fluorophores (*cf.*  
647 Table 1), recommendations for the design of intravital multicolor TPM microscope setups,  
648 and a discussion about strategies of data post-processing. Taken together, this database and set  
649 of recommendations will help scientists design intravital TPM experiments focusing  
650 simultaneously on biological targets of diverse natures, ranging from cells (*e.g.*, neurons,  
651 astrocytes) and sub-cellular structures (*e.g.*, DNA, organelles) to functional processes – whose  
652 exploration greatly benefits from the optogenetics technology – (*e.g.*, ion transport, membrane  
653 potential), and through chemical or environmental parameters (*e.g.*, mechanical strains, pH)  
654 or even potentially connectomics (Nemoto et al. 2015).

655 Extending beyond the current trends in neuroscience, this comprehensive two-photon probe  
656 database, as well as the resources related to intravital multicolor TPM, is intended to broaden  
657 the use of TPM not only in neuroscience, but also to other fields of biology in which the full  
658 extent of this technology's capabilities has not yet been revealed.



## 659 **Figures**

660

661 **Fig. 1** Optical setup for multicolor two-photon microscopy

662 DC: dichroic mirror; NDD: non-descanned detector; OPO: optical parametric oscillator.

663 <sup>1</sup>: Dichroic mirror used to collect fluorescent photons on five non-descanned detectors.

664 Excitatory infrared photons pass through the mirror whereas fluorescence photons are

665 reflected. This mirror must be removed to collect photons in descanned mode.

666 <sup>2</sup>: Dichroic mirror used to collect fluorescent photons on a spectral chip in descanned mode.

667 Modified from (Ricard and Debarbieux 2014).

668

669 **Fig. 2** Wavelength mixing–fluorescence lifetime imaging of endogenous fluorophores

670 When the excitation beams  $\lambda_1 = 760$  nm and  $\lambda_2 = 1041$  nm are synchronized ( $\Delta t = 0$ ), a third

671 virtual wavelength for two-photon excitation  $\lambda_v = 879$  nm is created by wavelength mixing. A

672 time-correlated single photon counting system (TCSPC) is used to perform FLIM.

673 Originally published in *Scientific reports* (Stringari et al. 2017) under a Creative Commons

674 Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>).

675

676 **Fig. 3** Wavelength-mixing optimal combination of two sets of fluorophores

677 Two-photon excitation and emission spectra of two sets of fluorophores that can be efficiently

678 excited by wavelength mixing with  $\lambda_1 = 850$  nm and  $\lambda_2 = 1230$  nm (full arrows), resulting in

679  $\lambda_v = 1005$  nm (dashed arrow). (a) **CFP, eGFP, mOrange2, mKate2, eqFP670**. (b) **Hoechst,**

680 **eGFP, Kusabira Orange, CMTPX Red, Alexa 647, Atto 680**.

681 Originally published in *Scientific reports* (Rakhymzhan et al. 2017) under a Creative

682 Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>).

683

684 **Fig. 4** Example of similarity unmixing of more fluorochromes than available channels

685 (a) Images of cells labelled with several fluorophores, showing crosstalk in two detection

686 channel. (b) Extraction of a relative signal distribution in the two channels pixels by pixel,

687 corresponding to . (c) The algorithm calculates similarities between the known fingerprints of

688 each fluorophore and the signature of undefined fluorophores extracted during step (b). (d)

689 Color separation based on the ratios between the fluorophore fingerprints.

690 Originally published in *Scientific reports* (Rakhymzhan et al. 2017) under a Creative

691 Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>).

692

693 **Tables**

694 **Table 1** Routes of administration of fluorochromes for intravital two-photon microscopy of  
 695 brain cells, structures, and functions.

<b>Technique</b>	<b>Target</b>	<b>Probe</b>
Single-cell electroporation	<i>Diverse (e.g. neurons (Liu and Haas 2011))</i>	<i>Diverse</i>
<i>In utero</i> electroporation (Wang and Mei 2013)	<i>e.g. layer 2/3 cortical neurons (Saito and Nakatsuji 2001)</i>	<i>e.g. eGFP</i>
Intraperitoneal administration	Amyloid- $\beta$ plaques	<i>e.g. SAD1 (Heo et al. 2013)</i>
Intravenous administration	Astrocytes	<b>Sulforhodamine 101</b> (Nimmerjahn et al. 2004), <b>sulforhodamine B</b> (Vérant et al. 2013)
	Blood vessels	<i>e.g. Rhodamine B dextran, quantum-dots (Ricard et al. 2016a)</i>
	Arteries	<b>Alexa Fluor 633</b> (Shen et al. 2012)
	Amyloid- $\beta$ plaques	<i>e.g. DCIP-1 (Zhu et al. 2017)</i>
Viral transduction (Nassi et al. 2015)	Spatially-restricted ( <i>e.g. axonal domain of neurons (Tervo et al. 2016) and genetically-defined cells (e.g. oligodendrocytes (Powell et al. 2016))</i> )	<i>Diverse</i>
	Calcium activity	Genetically encoded Ca <sup>2+</sup> indicators (GECIs)
	Membrane potential (V <sub>m</sub> )	Genetically encoded voltage sensors (GEVs)
Whole-cell and bulk loading	Ion activity (neurons, astrocytes (Reeves et al. 2011))	Ion indicators ( <i>e.g. Fura-2-AM, Fluo-4-AM, Oregon Green BAPTA-1 (Garaschuk et al. 2006)</i> )
	Membrane potential (V <sub>m</sub> )	Voltage-sensitive dyes ( <i>e.g. ANNINE-6 (Kuhn et al. 2008), RH-1692 (Murphy et al. 2008)</i> )

696  
 697

698 **Table 2** Biophysical properties of two-photon–suitable non-specific probes : peak wavelength  
699 of two-photon action cross-section ( $\lambda_{2PA}$ ) ; peak two-photon action cross-section ( $\sigma_2\phi$ ) ; peak  
700 wavelength of molecular brightness ( $\lambda_{\epsilon_{max}}$ ) ; peak molecular brightness ( $\epsilon_{max}$ ) ; fluorescence  
701 wavelength ( $\lambda_{fluo}$ ). Probes discussed in the text are in bold.  
702 (1) : calculated from the  $\sigma_2$  and  $\phi$  values  
703 (2) : data from commercial provider  
704 \* : data from [http://www.drbio.cornell.edu/cross\\_sections.html](http://www.drbio.cornell.edu/cross_sections.html) (accessed 10/24/2017)

Probe	$\lambda_{2PA}$ [nm]	$\sigma_2\phi$ [GM]	$\lambda_{\epsilon_{max}}$ [nm]	$\epsilon_{max}$ [kcpm]	$\lambda_{fluo}$ [nm]	Comment	Reference
— Pond 2002 Derivative 1	830	1207 (1)			536	Hydrophobic	Figure 3(1) in (Pond et al. 2002)
— Pond 2002 Derivative 2	830	1427 (1)			528	Hydrophobic	Figure 3(2) in (Pond et al. 2002)
— Pond 2002 Derivative 3	790	2.67 (1)			504	Hydrophobic	Figure 3(3) in (Pond et al. 2002)
— Pond 2002 Derivative 4	825	11 (1)			510	Hydrophobic	Figure 3(4) in (Pond et al. 2002)
— Sadowski 2017 Derivative 4a	740	112			671		Figure 3 in (Sadowski et al. 2017)
— Sadowski 2017 Derivative 4b	820	83			699		Figure 3 in (Sadowski et al. 2017)
— Sadowski 2017 Derivative 4c	740	70			662		Figure 3 in (Sadowski et al. 2017)
— Sadowski 2017 Derivative 5a (7a)	720	1450			633		Figure 3 in (Sadowski et al. 2017)
— Sadowski 2017 Derivative 5b (7c)	720	500			643		Figure 3 in (Sadowski et al. 2017)
— Sadowski 2017 Derivative 5c (7d)	860	340			736		Figure 3 in (Sadowski et al. 2017)
5C-TMR	830	135	850	31.5	580 (2)		Figure 5 in (Mütze et al. 2012)
7-amino-4-	703 <				439 (2)		Figure 3 in

methylcoumarin	722 >> 863						(Bestvater et al. 2002)
Alexa Fluor 350	~715				442 (2)		Figure 5C in (Trägårdh et al. 2015)
Alexa Fluor 430	870	11.6	910	16	541 (2)		Figure 2 in (Mütze et al. 2012)
Alexa Fluor 488	940	~30			519 (2)		Figure 2 in (Anderson and Webb 2011)
Alexa Fluor 514			790, 960	~32, ~25	542 (2)		Figure 2 in (Mütze et al. 2012)
Alexa Fluor 546			820	58	573 (2)		Figure 2 in (Mütze et al. 2012)
Alexa Fluor 568			780	~25	603 (2)		Figure 2 in (Mütze et al. 2012)
Alexa Fluor 594			800	~32	617 (2)		Figure 2 in (Mütze et al. 2012)
Alexa Fluor 610			820	~28	628 (2)		Figure 2 in (Mütze et al. 2012)
<b>Alexa Fluor 633</b>			820	~24	647 (2)	Arteries (Shen et al. 2012)	Figure 2 in (Mütze et al. 2012)
Alexa Fluor 647	1240	~45			665 (2)	OPO-friendly	Figure 7 in (Kobat et al. 2009)
Alexa Fluor 680	1280	~75			702 (2)	OPO-friendly	Figure 7 in (Kobat et al. 2009)
Alexa Fluor 700	1300	~52			723 (2)	OPO-friendly	Figure 7 in (Kobat et al. 2009)
Aminomethyl coumarin acetate	700				445 (2)		– (Bok et al. 2015)
Ant-PHEA (in water)	800	2480			575 (2)		Figure 6 in (Mettra et al. 2016)
ATTO 590	790	~250 (NHS ester)			624 (2)	STED-compatible	Figure 2E in (Velasco et al. 2015)
ATTO 594	800	~220 (NHS ester), ~140 (antibodies)			627 (2)	STED-compatible	Figure 2D in (Velasco et al. 2015)
ATTO 647N	840	~270			669 (2)	STED-	Figure 2A in

		(NHS ester), ~120 (streptavidin)				compatible	(Velasco et al. 2015)
ATTO 680	~1260				695 (2)	OPO-friendly	Figure 2D in (Rakhymzhan et al. 2017)
BODIPY (in water)	920	17.12			Variable		*
BODIPY 492/515 (in water)	920	14.3	920	47.4	515		Figure 5 in (Mütze et al. 2012)
BODIPY-FL (in DMSO)	920 << 972				512 (2)		Figure 3 in (Bestvater et al. 2002)
BODIPY-TR	1080	242	1060	15.2	618 (2)		Figure 5 in (Mütze et al. 2012)
Brilliant Violet 421	710-1000				421		– (Figure 6) (Chattopadhyay et al. 2012)
<b>Cascade Blue</b>	740, 800	2.1 (at 750 nm)			420 (2)		Figure 7 in (Xu and Webb 1996)
CellTracker Blue	780				466 (2)		– (Zinselmeyer et al. 2009)
CellTracker Orange	820				565 (2)		– (Miller et al. 2002)
<b>CellTracker Red</b>	~1080				602 (2)		Figure 2D in (Rakhymzhan et al. 2017)
<b>CFP</b>	840	~180			485 (2)		Figure 3B in (Zipfel et al. 2003b)
CFSE	780				521 (2)		– (Miller et al. 2002)
Citrine	968	6.7			529 (Griesbeck et al. 2001)		Figure 1 in (Drobizhev et al. 2011)
Coumarin 307	800	15.3			490 (in ethanol) (2)		*
Cy2-IgG	837 < 905 > 981				505 (2)		Figure 4 in (Bestvater et al. 2002)
Cy3-IgG	1032				565 (2)		Figure 4 in (Bestvater et al. 2002)

Cy5.5	1280	~60			702 (2)	OPO-friendly	Figure 7 in (Kobat et al. 2009)
Dronpa-3	920				515	Reversibly switchable	– (Ando et al. 2007; Kao et al. 2012)
<b>dsRed</b>	> 990	108			583 (Shaner et al. 2008)		*
dsRed2	1050	73			587		Figure S1 in (Drobizhev et al. 2011)
E2-Crimson	1138	1.8			643		Figure S1 in (Drobizhev et al. 2011)
EBFP2.0	750	9.2			446		Figure 1 in (Drobizhev et al. 2011)
<b>eCFP</b>	857	12			476		Figure S1 in (Drobizhev et al. 2011)
<b>eGFP (pH 8)</b>	927	30			510		Figure S1 in (Drobizhev et al. 2011)
eqFP650	1112	8.5			646		Figure S1 in (Drobizhev et al. 2011)
<b>eqFP670</b>	1120	1.3			661		Figure S1 in (Drobizhev et al. 2011)
Evans Blue	850				680 (Saria and Lundberg 1983)		– (Bennewitz et al. 2014)
<b>eYFP</b>	960	25			527 (Merzlyak et al. 2007)		– (Table 2) (Blab et al. 2001)
<b>Fluorescein</b> (in water, pH = 11)	770	39	800	18.4	520 (Zhu et al. 2005)		Figure 5 in (Mütze et al. 2012)
Fluorescein isothiocyanate (FITC)	800				525 (2)		Figure 3B in (Wang and Yeh 2012)
<b>GFP</b>	800	6.5			504 (Chattoraj et al. 1996)		*
Hilyte Fluor 488	~815, 960	~55, ~30			525 (2)		Figure 2 in (Anderson and Webb 2011)
Katushka	1080	23			635		Figure S1 in

					(Shcherbo et al. 2007)		(Drobizhev et al. 2011)
Katushka2	1140	27			633 (2)	OPO-friendly	Figure S1 in (Drobizhev et al. 2011)
Kusabira Orange	~1110				561 (Karasawa et al. 2004)		Figure 2D in (Rakhymzhan et al. 2017)
Lissamine rhodamine	837 >> 1116				~580 (2)		Figure 4 in (Bestvater et al. 2002)
LSS-mKate1	920	~40			624		Figure 1E in (Piatkevich et al. 2010)
LSS-mKate2	920	~90			605		Figure 1E in (Piatkevich et al. 2010)
Lucifer Yellow	840	1.4			540 (2)		*
mAmetrine	809	40			526 (Ai et al. 2008)		Figure S1 in (Drobizhev et al. 2011)
mBanana	1070	44			553 (Shaner et al. 2004)	OPO-friendly	Figure S1 in (Drobizhev et al. 2011)
mCardinal	>1080				659	OPO-friendly	Figure S3 in (Chu et al. 2014)
mCerulean	840	~75			475-503 (Ai et al. 2006)		Figure S1 in (Drobizhev et al. 2011)
mCFP	840	187			475 (Shaner et al. 2005)		*
mCherry	1080 (Drobizhev et al. 2011), 1160 (Vadakkan et al. 2009)	6.4 (at 1080 nm)			610 (Shaner et al. 2008)	OPO-friendly	Figure S1 in (Drobizhev et al. 2011) and Figure 8 in (Vadakkan et al. 2009)
mCitrene	960	~320					Figure 3C in (Rizzo et al. 2006)
mCitrine (pH 8)	950	7.6			527		Figure S1 in (Drobizhev et al. 2011)
mEGFP	960	~300			507 (Shaner et al. 2005)		Figure 3B in (Rizzo et al. 2006)
mGrape3	1140	1.6			645		Figure S1 in (Drobizhev



							et al. 2011)
mKate (pH 8)	1118	14			635 (Shcherbo et al. 2007)	OPO-friendly	Figure S1 in (Drobizhev et al. 2011)
<b>mKate2</b>	1140	30			633 (Shcherbo et al. 2009)	OPO-friendly	Figure S1 in (Drobizhev et al. 2011)
mKeima	~880	~70			620		Figure 1E in (Piatkevich et al. 2010)
mNeptune	1104	12			651 (Chu et al. 2014)	OPO-friendly	Figure S1 in (Drobizhev et al. 2011)
mOrange	1080	47			565	OPO-friendly	Figure S1 in (Drobizhev et al. 2011)
<b>mOrange2</b>	~1080				565 (Shaner et al. 2008)		Figure 2B in (Rakhymzhan et al. 2017)
mPlum	1105	2.9			644		Figure S1 in (Drobizhev et al. 2011)
mRaspberry	1118	5.8			625 (Shcherbo et al. 2007)	OPO-friendly	Figure S1 in (Drobizhev et al. 2011)
mRFP	1080	13			611		Figure S1 in (Drobizhev et al. 2011)
mStrawberry	1070	6.8			596 (Shaner et al. 2004)		Figure S1 in (Drobizhev et al. 2011)
mTangerine	1055	3.3			584		Figure S1 in (Drobizhev et al. 2009, 2011)
mVenus	960	~200			525 (Sarkar et al. 2009)		Figure 3C in (Rizzo et al. 2006)
mWasabi	927	7.3			508		Figure S1 in (Drobizhev et al. 2011)
Near-iRFP	1260				713	OPO-friendly	Figure S5 in (Filonov et al. 2011)
Neptune	1120	16			647		Figure S1 in (Drobizhev et al. 2011)
Pacific Blue	780				455 (2)		– (Lukomska et al. 2006)

Peridinin chlorophyll	850				678		– (Bok et al. 2015)
Phycoerythrin	1064	322			576 (2)		– (Chen et al. 1997; So et al. 2000)
QD550 (organic)	700-1000	~2000			550	Broad absorption spectrum	Figure 1A in (Larson et al. 2003)
QD550 (water-soluble)	700-1000	~2000			550	Broad absorption spectrum	Figure 1A in (Larson et al. 2003)
QD567 (water-soluble)	700-1000	~10000			567	Broad absorption spectrum	Figure 1A in (Larson et al. 2003)
QD605 (water-soluble)	700-1000	47000			605	Broad absorption spectrum	Figure 1A in (Larson et al. 2003)
QD630 (organic)	700-1000	~2000			630	Broad absorption spectrum	Figure 1A in (Larson et al. 2003)
Resorufin	1040	9	1060	33.9	585 (2)		Figure 5 in (Mütze et al. 2012)
Rhodamine 110	790	48	800	30.9	521 (2)		Figure 5 in (Mütze et al. 2012)
Rhodamine 6G	700, ~820	~150, ~40			~570 (Zehentbauer et al. 2014)		Figure 1C in (Albota et al. 1998)
<b>Rhodamine B</b>	830	204			590 (2)		*
Rhodamine Green	850				527 (2)		Figure 3 in (Heinze et al. 2000)
Sapphire	810	40			511 (Zapata-Hommer and Griesbeck 2003)		*
SeTa-632	820	200			641 (2)	Broad absorption spectrum	Figure 1B in (Podgorski et al. 2012)
SeTa-646	840	500			656 (2)	Broad absorption spectrum	Figure 1B in (Podgorski et al. 2012)
SeTa-660	840	1500			672 (2)	Broad absorption spectrum	Figure 1B in (Podgorski et al. 2012)
SeTa-670	840	2000			688 (2)	Broad absorption spectrum	Figure 1B in (Podgorski et al. 2012)
SeTa-700	900	200			703 (2)	Broad absorption	Figure 1B in (Podgorski et al. 2012)

						spectrum	et al. 2012)
SeTau-647	920	3000			694 (2)	Broad absorption spectrum	Figure 2A in (Podgorski et al. 2012)
SeTau-665	900	9000			712 (2)	Broad absorption spectrum	Figure 1B in (Podgorski et al. 2012)
Silicon Rhodamine	830	~140 (antibodies)			~670	STED-compatible	Figure 2B in (Velasco et al. 2015)
STAR 635P	820	~110 (NHS carbonate)			651 (2)	STED-compatible	Figure 2C in (Velasco et al. 2015)
TagGFP2	896	27			506 (2)		Figure S1 in (Drobizhev et al. 2011)
TagRFP	1050	42			584 (Shaner et al. 2008)		Figure S1 in (Drobizhev et al. 2011)
tdKatushka2	1100	63			633 (Shcherbo et al. 2009)	OPO-friendly	Figure S1 in (Drobizhev et al. 2011)
tdRFP	1110	13.7 (1)			579	OPO-friendly	Figure 2B in (Herz et al. 2010)
<b>tdTomato</b>	1050	200			581 (Shaner et al. 2008)	OPO-friendly	Figure S1 in (Drobizhev et al. 2011)
Tetramethylrhodamine (TRITC)	840				576 (2)		Figure 3C in (Wang and Yeh 2012)
Texas Red	780				615 (Cahalan et al. 2002)		Figure 3 in (Heinze et al. 2000)
<b>Venus</b>	~965, ~1015	~19, ~15			528 (Nagai et al. 2002)		Figure 7B in (Hashimoto et al. 2010)
VivoTag 680	820				688		– (Swirski et al. 2007)
<b>YFP</b>	960	228			527		*

705

706

707 **Table 3** Biophysical properties of two-photon–suitable probes specific of brain cells and  
708 structures: peak wavelength of two-photon action cross-section ( $\lambda_{2PA}$ ) ; peak two-photon  
709 action cross-section ( $\sigma_2\phi$ ) ; peak wavelength of molecular brightness ( $\lambda_{\epsilon_{max}}$ ) ; peak molecular  
710 brightness ( $\epsilon_{max}$ ) ; fluorescence wavelength ( $\lambda_{fluo}$ ). Probes discussed in the text are in bold.  
711 (1) : calculated from the  $\sigma_2$  and  $\phi$  values  
712 (2) : data from commercial provider

Probe	$\lambda_{2PA}$ [nm]	$\sigma_2\phi$ [GM]	$\lambda_{\epsilon_{max}}$ [nm]	$\epsilon_{max}$ [kcp/sm]	$\lambda_{fluo}$ [nm]	Comment	Reference
<b>Alexa Fluor 633</b>			820	~24	647 (2)	Arteries (Shen et al. 2012)	Figure 2 in (Mütze et al. 2012)
Ant2-PHEA (in water)	800	490			654	Endothelium	Figure 6 in (Mettra et al. 2016)
<b>Sulforhodamine 101</b>	910	118	900	38.4	~605 (2)	Astroglia (Nimmerjahn et al. 2004)	Figure 5 in (Mütze et al. 2012)
<b>Sulforhodamine B</b>	810				~586 (2)	Astroglia	Figure 2A in (Appaix et al. 2012)

713  
714

715 **Table 4** Biophysical properties of two-photon–suitable environment sensing probes: peak  
716 wavelength of two-photon action cross-section ( $\lambda_{2PA}$ ) ; peak two-photon action cross-section  
717 ( $\sigma_2\phi$ ) ; fluorescence wavelength ( $\lambda_{fluo}$ ). Probes discussed in the text are in bold.  
718 (1) : calculated from the  $\sigma_2$  and  $\phi$  values  
719 (2) : data from commercial provider

Probe	$\lambda_{2PA}$ [nm]	$\sigma_2\phi$ [GM]	$\lambda_{fluo}$ [nm]	Comment	Reference
— Chen 2017 Probe L1 (pH 6.73 / pH 4.33)	710	135 / 68 (1)	460 / 580	Ratiometric pH indicator	Figure 6A in (Chen et al. 2017)
— Chen 2017 Probe L2 (pH 5.53 / pH 2.99)	700	67 / 110 (1)	465 / 540	Ratiometric pH indicator	Figure 6B in (Chen et al. 2017)
C-Laurdan (in EtOH)	780 (820)	64.5 (1)	487	Mechanical strain in the cell membrane	Figure S7 in (Kim et al. 2007)
Laurdan (in EtOH)	780	60 (1)	494	Mechanical strain in the cell membrane	Figure S7 in (Kim et al. 2007)
NP1 (in DMF)	740	155	400-500 / 600-750	Ratiometric pH indicator	Figure S4 in (Park et al. 2012)
SNARF-1	< 837		580-640 (2)	pH indicator between pH 7 and pH 8	Figure 3 in (Bestvater et al. 2002)
Thiophene DiHemiCyanine	740	~1.4 (with high-viscosity)	~590	Viscosity-sensitive dye	Figure 5A in (Baek et al. 2016)

720  
721

722 **Table 5** Biophysical properties of two-photon–suitable probes in the diseased brain: peak  
723 wavelength of two-photon action cross-section ( $\lambda_{2PA}$ ) ; peak two-photon action cross-section  
724 ( $\sigma_2\phi$ ) ; fluorescence wavelength ( $\lambda_{fluo}$ ). Probes discussed in the text are in bold.  
725 (1) : calculated from the  $\sigma_2$  and  $\phi$  values  
726 (2) : data from commercial provider

Probe	$\lambda_{2PA}$ [nm]	$\sigma_2\phi$ [GM]	$\lambda_{fluo}$ [nm]	Comment	Reference
Calcofluor White	590		430 (2)	Cellulose and chitin binding (identification of fungi and yeast)	Figure 5B in (Trägårdh et al. 2015)
DCIP-1 (in PBS / bound to amyloid- $\beta$ aggregates)	900	118	675 / 635	Amyloid- $\beta$ plaques, penetrates BBB	Figure S7 in (Zhu et al. 2017)
<b>Gatifloxacin</b>	700		~510	Fluoroquinolone antibiotics	Figure 1A in (Lee et al. 2016)
MeO-X04 (in PBS / EtOH)	720	10 / 75	452 / 444	Amyloid- $\beta$ plaques (Klunk et al. 2002)	– (Table S1) (Heo et al. 2013)
MNAH	820		536	Imaging of mitochondrial singlet oxygen	– (Liu et al. 2016)
<b>Moxifloxacin</b>	700		~530	Fluoroquinolone antibiotics	Figure 1A in (Lee et al. 2016)
PIB (in PBS / EtOH)	740	45 / 40	431 / 417	Amyloid- $\beta$ plaques	– (Table S1) (Heo et al. 2013)
<b>SAD1</b> (in PBS / EtOH)	750	10 / 170	497 / 465	Amyloid- $\beta$ plaques	– (Table S1) (Heo et al. 2013)

727  
728

729 **Table 6** Biophysical properties of two-photon–suitable functional probes : peak wavelength  
730 of two-photon action cross-section ( $\lambda_{2PA}$ ) ; peak two-photon action cross-section ( $\sigma_2\phi$ ) ; peak  
731 wavelength of molecular brightness ( $\lambda_{\epsilon_{max}}$ ) ; peak molecular brightness ( $\epsilon_{max}$ ) ; fluorescence  
732 wavelength ( $\lambda_{fluo}$ ). Probes discussed in the text are in bold.  
733 (1) : calculated from the  $\sigma_2$  and  $\phi$  values  
734 (2) : data from commercial provider  
735 \* : data from [http://www.drbio.cornell.edu/cross\\_sections.html](http://www.drbio.cornell.edu/cross_sections.html) (accessed 10/24/2017)  
736 # : data from [https://www.janelia.org/lab/harris-lab-apig/research/photophysics/two-photon-](https://www.janelia.org/lab/harris-lab-apig/research/photophysics/two-photon-fluorescent-probes)  
737 [fluorescent-probes](https://www.janelia.org/lab/harris-lab-apig/research/photophysics/two-photon-fluorescent-probes) (accessed 10/24/2017)

Probe	$\lambda_{2PA}$ [nm]	$\sigma_2\phi$ [GM]	$\lambda_{\epsilon_{max}}$ [nm]	$\epsilon_{max}$ [kcpsm]	$\lambda_{fluo}$ [nm]	Comment	Reference
<b>Calcium indicators</b>							
ACa1 (-Ca <sup>2+</sup> / +Ca <sup>2+</sup> )	– / 780	– / 110			498 / 498		Figure 2A in (Kim et al. 2008a)
ACa2 (-Ca <sup>2+</sup> / +Ca <sup>2+</sup> )	– / 780	– / 90			495 / 495		Figure 2A in (Kim et al. 2008a)
ACa3 (-Ca <sup>2+</sup> / +Ca <sup>2+</sup> )	– / 780	– / 95			500 / 517		Figure 2A in (Kim et al. 2008a)
ACaL (-Ca <sup>2+</sup> / +Ca <sup>2+</sup> )	– / 780	– / 90			500 / 502		Figure 1B in (Mohan et al. 2009)
ACaLN (-Ca <sup>2+</sup> / +Ca <sup>2+</sup> )	– / 750	– / 20			494 / 497		– (Table 1) (Lim et al. 2011a)
BCaM (-Ca <sup>2+</sup> / +Ca <sup>2+</sup> )	– / 780	– / 150			470 / 470		Figure 1C in (Kim et al. 2010a)
Cal-590	1050				590		Figure 1B in (Tischbirek et al. 2015)
Calcium Crimson (+Ca <sup>2+</sup> )	870	95			615 (2)		*
Calcium Green (+Ca <sup>2+</sup> )	820, 960	46, 57			531 (2)		*
Calcium Orange (+Ca <sup>2+</sup> )	820	60			576 (2)		*

CaRuby-Cl	912				604		Figure 5 in (Collot et al. 2012)
CaRuby-F	917				604		Figure 5 in (Collot et al. 2012)
CaRuby-Me	917				604		Figure 5 in (Collot et al. 2012)
Fluo-3	810	13			520-530 (Svoboda and Yasuda 2006)		*
<b>Fluo-4</b>	810		800, 930	~6, ~6	520-530 (Yasuda et al. 2004)		Figure 4 in (Mütze et al. 2012)
Fluo-4FF	810				516 (2)		– (Yasuda et al. 2004)
Fluo-5F	810				516 (2)		– (Sabatini et al. 2002)
Fluo-8			930, 1000	~4	514 (2)		Figure 4 in (Mütze et al. 2012)
<b>Fura-2</b> +Ca <sup>2+</sup> )	780	~35			505 (Cahalan et al. 2002)		Figure 1B in (Mohan et al. 2009)
GCaMP2			950	~6	511 (Tallini et al. 2006)		Figure 4 in (Mütze et al. 2012)
GCaMP3 (-Ca <sup>2+</sup> / +Ca <sup>2+</sup> )	980	0.126 / 0.33 *			515 (Chen et al. 2013)		Figure 4I in (Helassa et al. 2015)
GCaMP3bright (-Ca <sup>2+</sup> / +Ca <sup>2+</sup> )	950	0.02 / 0.7 *			~515	high fluorescence dynamic range	Figure 4I in (Helassa et al. 2015)
GCaMP3fast (-Ca <sup>2+</sup> / +Ca <sup>2+</sup> )	990	0.014 / 0.2 *			~515	fast Ca <sup>2+</sup> response times	Figure 4H in (Helassa et al. 2015)
GCaMP5A (pH 9.5)	~940			9.9	~515		– (Table 3) (Akerboom et al. 2012)
GCaMP5D (pH 9.5)	~940			9.0	~515		– (Table 3) (Akerboom et al. 2012)
GCaMP5G (pH 9.5)	~940			9.3	515 (Chen et al. 2013)		Figure 1D in (Akerboom et al. 2012)
GCaMP6f	~940	~35			515 (Chen et al. 2013)		#
GCaMP6s	940	20			515		Figure 5A in



					(Chen et al. 2013)		(Dana et al. 2016)
iGluSnFR	~940				~515	Glutamate release	Figure S13 in (Marvin et al. 2013)
<b>Indo-1</b>	730				490 / 405 (Cahalan et al. 2002)		* and Figure 3A in (Wang and Yeh 2012)
<b>jRCaMP1a</b>	~1070	~8			~593	OPO-friendly	Figure 2S1B in (Dana et al. 2016)
<b>jRCaMP1b</b>	~1080	~11			~593	OPO-friendly	Figure 2S1B in (Dana et al. 2016)
<b>jRGECO1a</b>	~1070	~7			~593	OPO-friendly	Figure 2S1B in (Dana et al. 2016)
K-GECO1 (-Ca <sup>2+</sup> / +Ca <sup>2+</sup> )	~1100	~10			594 / 590		Figure 2C in (Shen et al. 2018)
mApple	1070			7.0	592		– (Table 3) (Akerboom et al. 2013)
mRuby	1060			4.0	590		– (Table 3) (Akerboom et al. 2013)
<b>Oregon Green BAPTA-1</b>	800	24			523		Figure 2A in (Kim et al. 2008a)
<b>RCaMP1a</b> (-Ca <sup>2+</sup> / +Ca <sup>2+</sup> )	1070 / 1070			– / 5.9	594 / 595		– (Table 3) (Akerboom et al. 2013)
RCaMP1c (-Ca <sup>2+</sup> / +Ca <sup>2+</sup> )	1070 / 1070			– / 7.3	597 / 595		– (Table 3) (Akerboom et al. 2013)
RCaMP1d (-Ca <sup>2+</sup> / +Ca <sup>2+</sup> )	1070 / 1070			– / 7.7	597.5 / 592.5		– (Table 3) (Akerboom et al. 2013)
RCaMP1f (-Ca <sup>2+</sup> / +Ca <sup>2+</sup> )	1070 / 1070			– / 8.2	597 / 591.5		Figure 2F in (Akerboom et al. 2013)
RCaMP1h	~1070	~27			595		#
R-GECO1 (-Ca <sup>2+</sup> / +Ca <sup>2+</sup> )	1065 / 1065			– / 3.8	598 / 588		– (Table 3) (Akerboom et al. 2013)
<b>Other ions</b>							
6-CO2H-ZAP4 (-Zn <sup>2+</sup> / +Zn <sup>2+</sup> )	<700, >1000	– / 86			527 / 523	Zinc	Figure 2B in (Khan et al. 2014)
ANa1 (+Na <sup>+</sup> )	780	95			500	Sodium	Figure 1C in (Kim et al.

							2010b)
<b>Asante NaTRIUM Green-2 (ANG-2) (-Na<sup>+</sup> / +Na<sup>+</sup>)</b>	780	0.84 / 5.7			542	Sodium	Figure 2 in (Roder and Hille 2014)
AZn1 (-Zn <sup>2+</sup> / +Zn <sup>2+</sup> )	- / 780	- / 95			496 / 498	Zinc	Figure 1B in (Kim et al. 2008b)
AZn2 (-Zn <sup>2+</sup> / +Zn <sup>2+</sup> )	- / 780	- / 110			494 / 499	Zinc	Figure 1B in (Kim et al. 2008b)
AZnE1 (-Zn <sup>2+</sup> / +Zn <sup>2+</sup> )	- / 780	- / 86			502 / 503	Zinc	Figure 2A in (Danish et al. 2011)
AZnE2 (-Zn <sup>2+</sup> / +Zn <sup>2+</sup> )	- / 780	- / 86			503 / 504	Zinc	Figure 2A in (Danish et al. 2011)
AZnM1 (-Zn <sup>2+</sup> / +Zn <sup>2+</sup> )	- / 780	- / 88			501 / 504	Zinc	Figure 2A in (Danish et al. 2011)
AZnM2 (-Zn <sup>2+</sup> / +Zn <sup>2+</sup> )	- / 780	- / 86			504 / 504	Zinc	Figure 2A in (Danish et al. 2011)
AZnN (-Zn <sup>2+</sup> / +Zn <sup>2+</sup> )	- / 780	- / 89			500 / 502	Zinc	Figure 2A in (Danish et al. 2011)
FMg1 (-Mg <sup>2+</sup> / +Mg <sup>2+</sup> )	- / 740	- / 87			540 / 540	Magnesium	Figure 1C in (Dong et al. 2012)
FMg2 (-Mg <sup>2+</sup> / +Mg <sup>2+</sup> )	- / 740	- / 76			555 / 555	Magnesium	Figure 1C in (Dong et al. 2012)
NC7	860	77 (1)			520	Magnesium	Figure 4 in (Yin et al. 2015)
OC7	740	71 (1)			500	Magnesium	Figure 4 in (Yin et al. 2015)
PhenGreen-FL	1074				517 (Petrat et al. 1999)	Heavy metals	Figure 3 in (Bestvater et al. 2002)
P-Zn (-Zn <sup>2+</sup> / +Zn <sup>2+</sup> )	700 / 700	304 / 565 (1)			465 / 550	Zinc	Figure 1A in (Li et al. 2017)
SBFI (+Na <sup>+</sup> )	780	20			539	Sodium	Figure 1C in (Kim et al. 2010b)
Sodium Green(+Na <sup>+</sup> )	800	30			532	Sodium	Figure 1C in (Kim et al. 2010b)
SZn-Mito (-Zn <sup>2+</sup> / +Zn <sup>2+</sup> )	- / 760	- / 75			500 / 493	Zinc	Figure 1B in (Masanta et al. 2011)
SZn2-Mito (-	- /	- /			536 / 536	Zinc	Figure 1B in

Zn <sup>2+</sup> / +Zn <sup>2+</sup> )	750	155					(Baek et al. 2012)
SZnC (-Zn <sup>2+</sup> / +Zn <sup>2+</sup> )	750 / 750	16 / 92			499 / 499	Zinc (Golgi-localized)	Figure 2D in (Singh et al. 2015)
— Schwarze 2015 Probe 1 (DMSO / K <sup>+</sup> / Na <sup>+</sup> )	860	3.1 / 9.4 / 3.4			511	Potassium / Sodium	Figure 2B in (Schwarze et al. 2015)
— Schwarze 2015 Probe 2 (DMSO / K <sup>+</sup> )	840	5.7 / 16.1			493	Potassium	– (Table 1) (Schwarze et al. 2015)
<b>Voltage-sensitive dyes</b>							
<b>ANNINE-6</b>	1020				560-660 (Frey et al. 2006)		– (Kuhn et al. 2008)
ANNINE-6plus	1060				560-660		– (Kuhn et al. 2004)
ArcLight A242	950				<775		– (Table 1) (Brinks et al. 2015)
ASAP1	950				<775		– (Table 1) (Brinks et al. 2015)
BP6	740	100			545	Mitochondrial membrane potential	Figure 7 in (Moritomo et al. 2014)
CAESR	968				<775		– (Table 1) (Brinks et al. 2015)
di-2-ANEP(F)PTEA	1060				632 (bound to lipids)		– (Yan et al. 2012)
<b>di-3-ANEPDPHQ</b>	850				>560		Figure 5A in (Fisher et al. 2008)
Di-4-ANEPPS	940	5			635 (in ethanol) (2)		*
Di-8-ANEPDPHQ	~900-950	~20			620 (in octanol)		Figure 3 in (Fisher et al. 2005)
Di-8-ANEPPS	940	10			625 (in octanol)		* and Figure 5 in (Fisher et al. 2005)
FlicR1	1120				597	OPO-friendly	– (Abdelfattah et al. 2016)
Merocyanine 540	>950	4.4 (at 960 nm)			615 (in octanol)		Figure 3 in (Fisher et al. 2005)

Nile Blue A	<800	0.6 (at 800 nm)			660 (in octanol)		Figure 3 in (Fisher et al. 2005)
QuasAr1	1200				660-775		– (Table 1) (Brinks et al. 2015)
QuasAr2	1200				660-775		Figure S3 in (Brinks et al. 2015)
<b>RH-1692</b>	~800- 850	1.5 (at 800 nm)			680 (in octanol)		Figure 3 in (Fisher et al. 2005)
RH-237	<800, >960	8.9 (at 800 nm)			676 (in octanol)		Figure 3 in (Fisher et al. 2005)
RH-414	~950	12 (at 960 nm)			636 (in octanol)		Figure 3 in (Fisher et al. 2005)
RH-421	~950	16 (at 960 nm)			648 (in octanol)		Figure 3 in (Fisher et al. 2005)
RH-795	~950	10 (at 960 nm)			640 (in octanol)		Figure 3 in (Fisher et al. 2005)

738

739

740 **Table 7** Biophysical two-photon properties of intrinsic fluorophores : peak wavelength of  
741 two-photon action cross-section ( $\lambda_{2PA}$ ) ; peak two-photon action cross-section ( $\sigma_2\phi$ ) ; peak  
742 wavelength of molecular brightness ( $\lambda_{\epsilon_{max}}$ ) ; peak molecular brightness ( $\epsilon_{max}$ ) ; fluorescence  
743 wavelength ( $\lambda_{fluo}$ ). Probes discussed in the text are in bold.

<b>Probe</b>	$\lambda_{2PA}$ [nm]	$\sigma_2\phi$ [GM]	$\lambda_{fluo}$ [nm]	<b>Comment</b>	<b>Reference</b>
<b>Cholecalciferol</b>	<700, 820, 980	0.0006 (at 700 nm)	460	Vitamin D3	Figure 1A in (Zipfel et al. 2003a)
<b>FAD</b>	~880	<1	~530		Figure 1B in (Stringari et al. 2017)
<b>Folic acid</b>	<700	0.007 (at 700 nm)	~445	Vitamin B9	Figure 1A in (Zipfel et al. 2003a)
<b>NADH</b>	700	0.09 (at 700 nm)	~470		Figure 1A in (Zipfel et al. 2003a)
<b>Pyridoxine</b>	<700	0.008 (at 700 nm)	~390		Figure 1A in (Zipfel et al. 2003a)
<b>Retinol</b>	<700	0.07 (at 700 nm)	~490		Figure 1A in (Zipfel et al. 2003a)
<b>Riboflavin</b>	<700	0.8 (at 700 nm)	~530		Figure 1A in (Zipfel et al. 2003a)

744  
745

746 **Table 8** Biophysical properties of two-photon–suitable probes specific of sub-cellular  
747 structures : peak wavelength of two-photon action cross-section ( $\lambda_{2PA}$ ) ; peak two-photon  
748 action cross-section ( $\sigma_2\phi$ ) ; peak wavelength of molecular brightness ( $\lambda_{\epsilon_{max}}$ ) ; peak molecular  
749 brightness ( $\epsilon_{max}$ ) ; fluorescence wavelength ( $\lambda_{fluo}$ ). Probes discussed in the text are in bold.  
750 (1) : calculated from the  $\sigma_2$  and  $\phi$  values  
751 (2) : data from commercial provider  
752 \* : data from [http://www.drbio.cornell.edu/cross\\_sections.html](http://www.drbio.cornell.edu/cross_sections.html) (accessed 10/24/2017)

Probe	$\lambda_{2PA}$ [nm]	$\sigma_2\phi$ [GM]	$\lambda_{fluo}$ [nm]	Comment	Reference
Acridine Orange	837 > 882 >> 981		526 (DNA), 650 (RNA) (2)	Nucleic acids	Figure 3 in (Bestvater et al. 2002)
ANO1	750	170	502	Nitric oxide	Figure 3 in (Seo et al. 2012)
AS1	780	85	498	Glucose	Figure S6A in (Lim et al. 2012)
ASiR1 (in CH <sub>3</sub> CN)	750	258	593	Lysosomes	Figure S2 in (Zhang et al. 2018)
ASiR2 (in CH <sub>3</sub> CN)	760	237	601	Lysosomes	Figure S2 in (Zhang et al. 2018)
ASiR3 (in CH <sub>3</sub> CN)	760	160	606	Lysosomes	Figure S2 in (Zhang et al. 2018)
ASiR4 (in CH <sub>3</sub> CN)	760	179	608	Lysosomes	Figure S2 in (Zhang et al. 2018)
ASiR5 (in CH <sub>3</sub> CN)	750	189	609	Lysosomes	Figure S2 in (Zhang et al. 2018)
ASiR6 (in CH <sub>3</sub> CN)	750	193	601	Lysosomes	Figure S2 in (Zhang et al. 2018)
ASS (H <sub>2</sub> O / 2-AET)	780	11 / 113	457 / ~505	Thiols	Figure 1D in (Lee et al. 2010)
BLT-blue	750	160	451	Lysosomes	Figure 1B in (Han et al. 2012)
CAEI	850	152	550	Mitochondria	Figure 1B in (Miao et al. 2014)

CAI	860	328	562	Mitochondria	Figure 1B in (Miao et al. 2014)
CLT-blue	750	50	471	Lysosomes	Figure 1B in (Son et al. 2011)
CLT-yellow	850	47	549	Lysosomes	Figure 1B in (Son et al. 2011)
C-Laurdan (in EtOH)	780	64.5 (1)	487	Lipid rafts	Figure S7 in (Kim et al. 2007)
DAPI	580 >> 685		461 (2)	Nucleic acids	Figure 2A in (Trägårdh et al. 2015)
DCF2 (H <sub>2</sub> DCFDA) (in DMSO)	1065		538 (Yi et al. 2009)	Organelle tracker	Figure 3 in (Bestvater et al. 2002)
DiA	880 (study limited to [830-920 nm])		590	Lipophilic Tracer	– (Table 1) (Ruthazer and Cline 2002)
DiD	830		665	Lipophilic Tracer	– (Table 1) (Ruthazer and Cline 2002)
DiI	<700, 1020	96, 10	565 (Cahalan et al. 2002)	Lipophilic Tracer, organelles	*
DiO	880 (study limited to [830-920 nm])		501	Lipophilic Tracer	– (Table 1) (Ruthazer and Cline 2002)
ER-Tracker white/blue	728		430-640 (2)	Endoplasmic Reticulum (environmentally-sensitive)	Figure 5 in (Bestvater et al. 2002)
FMT-green	750	175	523	Mitochondria	Figure 1B in (Han et al. 2012)
Hoechst 33258	560 >> 717		461 (2)	Nucleic acids	Figure 5A in (Trägårdh et al. 2015)
Hoechst 33342	660 > 715		461 (2)	Nucleic acids	Figure 3A in (Bestvater et al. 2002)
Laurdan (in EtOH)	780	60 (1)	494	Lipid rafts	Figure S7 in (Kim et al. 2007)
LysoTracker Red	1010 < 1100		590 (2)	Organelle tracker	Figure 5 in (Bestvater et al. 2002)
LysoTracker Yellow	972		535 (2)	Organelle tracker	Figure 5 in (Bestvater et al. 2002)

MitoTracker Red	860, 1133	3.12 (at 860 nm)	599 (2)	Organelle tracker	Figure 5 in (Bestvater et al. 2002)
PKH26	950		567 (2)	Membrane labelling	– (Takaki et al. 2015)
PKH67	870, 850		502 (2)	Membrane labelling	– (Morelli et al. 2003; Kuwashima et al. 2005)
Propidium iodide	989 > 1015 >> 1099		617 (2)	Nucleic acids (dead cells)	Figure 3 in (Bestvater et al. 2002)
<b>Quinacrine</b>	678 > 697		525 (2)	Nucleic acids, therapeutic effect	Figure 3 in (Bestvater et al. 2002)
SL2 (in EtOH / THF)	800	40 / 185	576 / 495	Lipid rafts, turn-on	Figure 1B in (Lim et al. 2011b)
Rhodamine 123	913 > 1090		527 (2)	Mitochondria	Figure 3 in (Bestvater et al. 2002)
SYBR Gold	~820		~537 (2)	Nucleic acids	Figure 3 in (Neu et al. 2002)
SYBR Green	~820		520 (2)	Nucleic acids	Figure 3 in (Neu et al. 2002)
Syto 13	~780-800		509 (DNA), 514 (RNA) (2)	Nucleic acids	Figure 2 in (Neu et al. 2002)
Syto 40	900		441 (2)	Nucleic acids	Figure 2 in (Neu et al. 2002)
Syto 9	<800		503 (2)	Nucleic acids	Figure 2 in (Neu et al. 2002)
Sytox Blue	<800		480 (2)	Nucleic acids	Figure 3 in (Neu et al. 2002)
Sytox Green	~715		523 (2)	Nucleic acids	Figure 5C in (Trägårdh et al. 2015)
TO-PRO-3	1110		661 (2)	Nucleic acids, OPO-friendly	– (Smith et al. 2012)



754 **Table S1** Biophysical properties of two-photon–suitable probes in alphabetical order: peak  
755 wavelength of two-photon action cross-section ( $\lambda_{2PA}$ ) ; peak two-photon action cross-section  
756 ( $\sigma_2\phi$ ) ; peak wavelength of molecular brightness ( $\lambda_{\epsilon_{max}}$ ) ; peak molecular brightness ( $\epsilon_{max}$ ) ;  
757 fluorescence wavelength ( $\lambda_{fluo}$ ).

758 (1) : calculated from the  $\sigma_2$  and  $\phi$  values

759 (2) : data from commercial provider

760

761

## 762 **Compliance with Ethical Standards**

763 **Funding:** CH is supported by a F30 Fellowship (NS093719 from NIH NINDS), as well as the  
764 UCLA Medical Scientist Training Program (NIH NIGMS training grant GM08042). CP-C is  
765 supported by W81XWH-14-1-0433 (USAMRMC, DOD), a Developmental Disabilities  
766 Translational Research Program grant (#20160969 from John Merck), SFARI Awards 295438  
767 and 513155CP (Simons Foundation) and 5R01HD054453 (NICHD/NIH). GL is supported by  
768 the Agence Nationale de la Recherche (ANR-15-CE37-0004 “SmellBrain” & ANR-15-  
769 NEUC-0004 “Circuit\_OPL”).

770 **Conflict of Interest:** GL is supported by the life insurance company AG2R-La-Mondiale  
771 (“Vivons vélo pour l’Institut Pasteur”).

772 **Ethical approval:** All procedures performed in studies involving animals were in accordance  
773 with the ethical standards of the institution at which the studies were conducted. This article  
774 does not contain any studies with human participants performed by any of the authors.

775

## 776 **Bibliography**

- 777 Abdelfattah AS, Farhi SL, Zhao Y, et al (2016) A Bright and Fast Red Fluorescent Protein  
778 Voltage Indicator That Reports Neuronal Activity in Organotypic Brain Slices. *J Neurosci*  
779 36:2458–72 . doi: 10.1523/JNEUROSCI.3484-15.2016
- 780 Acker CD, Yan P, Loew LM (2011) Single-voxel recording of voltage transients in dendritic  
781 spines. *Biophys J* 101:L11-3 . doi: 10.1016/j.bpj.2011.06.021
- 782 Ai H, Hazelwood KL, Davidson MW, Campbell RE (2008) Fluorescent protein FRET pairs for  
783 ratiometric imaging of dual biosensors. *Nat Methods* 5:401–3 . doi:  
784 10.1038/nmeth.1207
- 785 Ai H, Henderson JN, Remington SJ, Campbell RE (2006) Directed evolution of a monomeric,  
786 bright and photostable version of *Clavularia cyan* fluorescent protein: structural  
787 characterization and applications in fluorescence imaging. *Biochem J* 400:531–40 . doi:  
788 10.1042/BJ20060874
- 789 Akemann W, Sasaki M, Mutoh H, et al (2013) Two-photon voltage imaging using a genetically  
790 encoded voltage indicator. *Sci Rep* 3:2231 . doi: 10.1038/srep02231
- 791 Akerboom J, Carreras Calderón N, Tian L, et al (2013) Genetically encoded calcium indicators  
792 for multi-color neural activity imaging and combination with optogenetics. *Front Mol*  
793 *Neurosci* 6:2 . doi: 10.3389/fnmol.2013.00002
- 794 Akerboom J, Chen T-W, Wardill TJ, et al (2012) Optimization of a GCaMP calcium indicator  
795 for neural activity imaging. *J Neurosci* 32:13819–40 . doi: 10.1523/JNEUROSCI.2601-  
796 12.2012
- 797 Albota MA, Xu C, Webb WW (1998) Two-Photon Fluorescence Excitation Cross Sections of  
798 Biomolecular Probes from 690 to 960 nm. *Appl Opt* 37:7352–6

799 Anderson VL, Webb WW (2011) Transmission electron microscopy characterization of  
800 fluorescently labelled amyloid  $\beta$  1-40 and  $\alpha$ -synuclein aggregates. *BMC Biotechnol*  
801 11:125 . doi: 10.1186/1472-6750-11-125

802 Ando R, Flors C, Mizuno H, et al (2007) Highlighted generation of fluorescence signals using  
803 simultaneous two-color irradiation on Dronpa mutants. *Biophys J* 92:L97-9 . doi:  
804 10.1529/biophysj.107.105882

805 Angevine JB, Sidman RL (1961) Autoradiographic study of cell migration during histogenesis  
806 of cerebral cortex in the mouse. *Nature* 192:766–768 . doi: 10.1038/192766b0

807 Appaix F, Girod S, Boisseau S, et al (2012) Specific in vivo staining of astrocytes in the whole  
808 brain after intravenous injection of sulforhodamine dyes. *PLoS One* 7:e35169 . doi:  
809 10.1371/journal.pone.0035169

810 Baek NY, Heo CH, Lim CS, et al (2012) A highly sensitive two-photon fluorescent probe for  
811 mitochondrial zinc ions in living tissue. *Chem Commun (Camb)* 48:4546–8 . doi:  
812 10.1039/c2cc31077e

813 Baek Y, Park SJ, Zhou X, et al (2016) A viscosity sensitive fluorescent dye for real-time  
814 monitoring of mitochondria transport in neurons. *Biosens Bioelectron* 86:885–891 . doi:  
815 10.1016/j.bios.2016.07.026

816 Bayerl SH, Niesner R, Cseresnyes Z, et al (2016) Time lapse in vivo microscopy reveals distinct  
817 dynamics of microglia-tumor environment interactions-a new role for the tumor  
818 perivascular space as highway for trafficking microglia. *Glia* 64:1210–26 . doi:  
819 10.1002/glia.22994

820 Becker W, Su B, Holub O, Weisshart K (2011) FLIM and FCS detection in laser-scanning  
821 microscopes: increased efficiency by GaAsP hybrid detectors. *Microsc Res Tech* 74:804–  
822 11 . doi: 10.1002/jemt.20959

823 Bennewitz MF, Watkins SC, Sundd P (2014) Quantitative intravital two-photon excitation  
824 microscopy reveals absence of pulmonary vaso-occlusion in unchallenged Sickle Cell  
825 Disease mice. *IntraVital* 3:e29748 . doi: 10.4161/intv.29748

826 Bestvater F, Spiess E, Stobrawa G, et al (2002) Two-photon fluorescence absorption and  
827 emission spectra of dyes relevant for cell imaging. *J Microsc* 208:108–115 . doi:  
828 10.1046/j.1365-2818.2002.01074.x

829 Blab GA, Lommerse PH., Cognet L, et al (2001) Two-photon excitation action cross-sections  
830 of the autofluorescent proteins. *Chem Phys Lett* 350:71–77 . doi: 10.1016/S0009-  
831 2614(01)01282-9

832 Bok S, Wang T, Lee C-J, et al (2015) In vivo imaging of activated microglia in a mouse model  
833 of focal cerebral ischemia by two-photon microscopy. *Biomed Opt Express* 6:3303–12 .  
834 doi: 10.1364/BOE.6.003303

835 Bonnin A, Torii M, Wang L, et al (2007) Serotonin modulates the response of embryonic  
836 thalamocortical axons to netrin-1. *Nat Neurosci* 10:588–597 . doi: 10.1038/nn1896

837 Borrell V, Yoshimura Y, Callaway EM (2005) Targeted gene delivery to telencephalic  
838 inhibitory neurons by directional in utero electroporation. *J Neurosci Methods*  
839 143:151–158 . doi: 10.1016/j.jneumeth.2004.09.027

840 Brain KL, Bennett MR (1997) Calcium in sympathetic varicosities of mouse vas deferens  
841 during facilitation, augmentation and autoinhibition. *J Physiol* 502:521–536 . doi:  
842 10.1111/j.1469-7793.1997.521bj.x

843 Brenner MH, Cai D, Swanson JA, Ogilvie JP (2013) Two-photon imaging of multiple  
844 fluorescent proteins by phase-shaping and linear unmixing with a single broadband  
845 laser. *Opt Express* 21:17256–64 . doi: 10.1364/OE.21.017256

846 Brenowitz SD, Regehr WG (2014) Presynaptic calcium measurements using bulk loading of

847 acetoxymethyl indicators. *Cold Spring Harb Protoc* 2014:750–7 . doi:  
848 10.1101/pdb.prot081828

849 Brinks D, Klein AJ, Cohen AE (2015) Two-Photon Lifetime Imaging of Voltage Indicating  
850 Proteins as a Probe of Absolute Membrane Voltage. *Biophys J* 109:914–21 . doi:  
851 10.1016/j.bpj.2015.07.038

852 Büning H, Huber A, Zhang L, et al (2015) Engineering the AAV capsid to optimize vector-host-  
853 interactions. *Curr Opin Pharmacol* 24:94–104 . doi: 10.1016/j.coph.2015.08.002

854 Burgold S, Bittner T, Dorostkar MM, et al (2011) In vivo multiphoton imaging reveals gradual  
855 growth of newborn amyloid plaques over weeks. *Acta Neuropathol* 121:327–35 . doi:  
856 10.1007/s00401-010-0787-6

857 Cahalan MD, Parker I, Wei SH, Miller MJ (2002) Two-photon tissue imaging: seeing the  
858 immune system in a fresh light. *Nat Rev Immunol* 2:872–880 . doi: 10.1038/nri935

859 Caviness VS, Takahashi T (1995) Proliferative events in the cerebral ventricular zone. *Brain*  
860 *Dev* 17:159–163 . doi: 10.1016/0387-7604(95)00029-B

861 Chaigneau E, Oheim M, Audinat E, Charpak S (2003) Two-photon imaging of capillary blood  
862 flow in olfactory bulb glomeruli. *Proc Natl Acad Sci U S A* 100:13081–6 . doi:  
863 10.1073/pnas.2133652100

864 Chattopadhyay PK, Gaylord B, Palmer A, et al (2012) Brilliant violet fluorophores: a new class  
865 of ultrabright fluorescent compounds for immunofluorescence experiments. *Cytometry*  
866 *A* 81:456–66 . doi: 10.1002/cyto.a.22043

867 Chatteraj M, King BA, Bublitz GU, Boxer SG (1996) Ultra-fast excited state dynamics in green  
868 fluorescent protein: multiple states and proton transfer. *Proc Natl Acad Sci U S A*  
869 93:8362–7

870 Chen Q, Cichon J, Wang W, et al (2012) Imaging neural activity using Thy1-GCaMP transgenic

871 mice. *Neuron* 76:297–308 . doi: 10.1016/j.neuron.2012.07.011

872 Chen S, Zhao M, Su J, et al (2017) Two novel two-photon excited fluorescent pH probes  
873 based on the A- $\pi$ -D- $\pi$ -A system for intracellular pH mapping. *Dye Pigment* 136:807–816  
874 . doi: 10.1016/j.dyepig.2016.09.020

875 Chen T-W, Wardill TJ, Sun Y, et al (2013) Ultrasensitive fluorescent proteins for imaging  
876 neuronal activity. *Nature* 499:295–300 . doi: 10.1038/nature12354

877 Chen Z, Kaplan DL, Yang K, et al (1997) Two-photon-induced fluorescence from the  
878 phycoerythrin protein. *Appl Opt* 36:1655–9

879 Chu J, Haynes RD, Corbel SY, et al (2014) Non-invasive intravital imaging of cellular  
880 differentiation with a bright red-excitable fluorescent protein. *Nat Methods* 11:572–8 .  
881 doi: 10.1038/nmeth.2888

882 Collot M, Loukou C, Yakovlev A V., et al (2012) Calcium rubies: A family of red-emitting  
883 functionalizable indicators suitable for two-photon Ca<sup>2+</sup> imaging. *J Am Chem Soc*  
884 134:14923–14931 . doi: 10.1021/ja304018d

885 Crowe SE, Ellis-Davies GCR (2014) Longitudinal in vivo two-photon fluorescence imaging. *J*  
886 *Comp Neurol* 522:1708–27 . doi: 10.1002/cne.23502

887 Cruz-Martin A, Crespo M, Portera-Cailliau C (2010) Delayed stabilization of dendritic spines  
888 in fragile X mice. *J Neurosci* 30:7793–7803

889 Dana H, Chen T-W, Hu A, et al (2014) Thy1-GCaMP6 Transgenic Mice for Neuronal  
890 Population Imaging In Vivo. *PLoS One* 9:e108697 . doi: 10.1371/journal.pone.0108697

891 Dana H, Mohar B, Sun Y, et al (2016) Sensitive red protein calcium indicators for imaging  
892 neural activity. *Elife* 5: . doi: 10.7554/eLife.12727

893 Danielyan A, Wu Y-W, Shih P-Y, et al (2014) Denoising of two-photon fluorescence images  
894 with block-matching 3D filtering. *Methods* 68:308–16 . doi:

895 10.1016/j.ymeth.2014.03.010

896 Danish IA, Lim CS, Tian YS, et al (2011) Two-photon probes for Zn<sup>2+</sup> ions with various  
897 dissociation constants. Detection of Zn<sup>2+</sup> ions in live cells and tissues by two-photon  
898 microscopy. *Chem Asian J* 6:1234–40 . doi: 10.1002/asia.201000720

899 Davis LM, Shen G (2007) Extension of multidimensional microscopy to ultrasensitive  
900 applications with maximum-likelihood analysis. In: Conchello J-A, Cogswell CJ, Wilson T  
901 (eds) *Proc. of SPIE*. p 64430N

902 Débarre D, Supatto W, Pena A-M, et al (2006) Imaging lipid bodies in cells and tissues using  
903 third-harmonic generation microscopy. *Nat Methods* 3:47–53 . doi: 10.1038/nmeth813

904 Deisseroth K (2015) Optogenetics: 10 years of microbial opsins in neuroscience. *Nat Neurosci*  
905 18:1213–25 . doi: 10.1038/nn.4091

906 Denk W, Strickler JH, Webb WW (1990) Two-photon laser scanning fluorescence microscopy.  
907 *Science* 248:73–6

908 Dimitrov D, He Y, Mutoh H, et al (2007) Engineering and characterization of an enhanced  
909 fluorescent protein voltage sensor. *PLoS One* 2:e440 . doi:  
910 10.1371/journal.pone.0000440

911 Dong X, Han JH, Heo CH, et al (2012) Dual-color imaging of magnesium/calcium ion activities  
912 with two-photon fluorescent probes. *Anal Chem* 84:8110–3 . doi: 10.1021/ac302210v

913 Dray N, Bedu S, Vuillemin N, et al (2015) Large-scale live imaging of adult neural stem cells in  
914 their endogenous niche. *Development* 142:3592–600 . doi: 10.1242/dev.123018

915 Drobizhev M, Makarov NS, Tillo SE, et al (2011) Two-photon absorption properties of  
916 fluorescent proteins. *Nat Methods* 8:393–9 . doi: 10.1038/nmeth.1596

917 Drobizhev M, Tillo S, Makarov NS, et al (2009) Absolute two-photon absorption spectra and  
918 two-photon brightness of orange and red fluorescent proteins. *J Phys Chem B* 113:855–



919 9 . doi: 10.1021/jp8087379

920 Ducros M, Moreaux L, Bradley J, et al (2009) Spectral unmixing: analysis of performance in  
921 the olfactory bulb in vivo. *PLoS One* 4:e4418 . doi: 10.1371/journal.pone.0004418

922 Ducros M, van 't Hoff M, van't Hoff M, et al (2011) Efficient large core fiber-based detection  
923 for multi-channel two-photon fluorescence microscopy and spectral unmixing. *J*  
924 *Neurosci Methods* 198:172–80 . doi: 10.1016/j.jneumeth.2011.03.015

925 Emiliani V, Cohen AE, Deisseroth K, Häusser M (2015) All-Optical Interrogation of Neural  
926 Circuits. *J Neurosci* 35:13917–26 . doi: 10.1523/JNEUROSCI.2916-15.2015

927 Eriksson A, Österroos A, Hassan S, et al (2015) Drug screen in patient cells suggests  
928 quinacrine to be repositioned for treatment of acute myeloid leukemia. *Blood Cancer J*  
929 5:e307 . doi: 10.1038/bcj.2015.31

930 Estrada G, Beetle C, Schummers J (2015) Simple method to improve spatial resolution for in  
931 vivo two-photon fluorescence imaging. *Appl Opt* 54:10044–50

932 Feng G, Mellor RH, Bernstein M, et al (2000) Imaging neuronal subsets in transgenic mice  
933 expressing multiple spectral variants of GFP. *Neuron* 28:41–51

934 Fenrich KK, Weber P, Hocine M, et al (2012) Long-term in vivo imaging of normal and  
935 pathological mouse spinal cord with subcellular resolution using implanted glass  
936 windows. *J Physiol* 590:3665–75 . doi: 10.1113/jphysiol.2012.230532

937 Fenrich KK, Weber P, Rougon G, Debarbieux F (2013) Long- and short-term intravital imaging  
938 reveals differential spatiotemporal recruitment and function of myelomonocytic cells  
939 after spinal cord injury. *J Physiol* 591:4895–902 . doi: 10.1113/jphysiol.2013.256388

940 Ferezou I, Bolea S, Petersen CCH (2006) Visualizing the cortical representation of whisker  
941 touch: voltage-sensitive dye imaging in freely moving mice. *Neuron* 50:617–29 . doi:  
942 10.1016/j.neuron.2006.03.043

943 Filonov GS, Piatkevich KD, Ting L-M, et al (2011) Bright and stable near-infrared fluorescent  
944 protein for in vivo imaging. *Nat Biotechnol* 29:757–61 . doi: 10.1038/nbt.1918

945 Fiole D, Deman P, Trescos Y, et al (2014) Two-photon intravital imaging of lungs during  
946 anthrax infection reveals long-lasting macrophage-dendritic cell contacts. *Infect Immun*  
947 82:864–72 . doi: 10.1128/IAI.01184-13

948 Fisher JAN, Barchi JR, Welle CG, et al (2008) Two-photon excitation of potentiometric probes  
949 enables optical recording of action potentials from mammalian nerve terminals in situ. *J*  
950 *Neurophysiol* 99:1545–53 . doi: 10.1152/jn.00929.2007

951 Fisher JAN, Salzberg BM, Yodh AG (2005) Near infrared two-photon excitation cross-sections  
952 of voltage-sensitive dyes. *J Neurosci Methods* 148:94–102 . doi:  
953 10.1016/j.jneumeth.2005.06.027

954 Frantsuzov PA, Volkán-Kacsó S, Jankó B (2013) Universality of the fluorescence intermittency  
955 in nanoscale systems: experiment and theory. *Nano Lett* 13:402–8 . doi:  
956 10.1021/nl3035674

957 Frey W, White JA, Price RO, et al (2006) Plasma membrane voltage changes during  
958 nanosecond pulsed electric field exposure. *Biophys J* 90:3608–15 . doi:  
959 10.1529/biophysj.105.072777

960 Fujii S, Hirota A, Kamino K (1981) Action potential synchrony in embryonic precontractile  
961 chick heart: optical monitoring with potentiometric dyes. *J Physiol* 319:529–41

962 Fukuchi-Shimogori T, Grove EA (2001) Neocortex Patterning by the Secreted Signaling  
963 Molecule FGF8. *Science* (80- ) 294:

964 Galli R, Uckermann O, Andresen EF, et al (2014) Intrinsic indicator of photodamage during  
965 label-free multiphoton microscopy of cells and tissues. *PLoS One* 9:e110295 . doi:  
966 10.1371/journal.pone.0110295

967 Garaschuk O, Milos R-I, Konnerth A (2006) Targeted bulk-loading of fluorescent indicators for  
968 two-photon brain imaging in vivo. *Nat Protoc* 1:380–6 . doi: 10.1038/nprot.2006.58

969 Gee KR, Brown KA, Chen WN, et al (2000) Chemical and physiological characterization of  
970 fluo-4 Ca(2+)-indicator dyes. *Cell Calcium* 27:97–106 . doi: 10.1054/ceca.1999.0095

971 Grienberger C, Chen X, Konnerth A (2014) NMDA Receptor-Dependent Multidendrite Ca(2+)  
972 Spikes Required for Hippocampal Burst Firing In Vivo. *Neuron* 81:1274–1281 . doi:  
973 10.1016/j.neuron.2014.01.014

974 Grienberger C, Konnerth A (2012) Imaging calcium in neurons. *Neuron* 73:862–85 . doi:  
975 10.1016/j.neuron.2012.02.011

976 Griesbeck O, Baird GS, Campbell RE, et al (2001) Reducing the environmental sensitivity of  
977 yellow fluorescent protein. Mechanism and applications. *J Biol Chem* 276:29188–94 .  
978 doi: 10.1074/jbc.M102815200

979 Grinvald A, Hildesheim R (2004) VSDI: a new era in functional imaging of cortical dynamics.  
980 *Nat Rev Neurosci* 5:874–85 . doi: 10.1038/nrn1536

981 Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca<sup>2+</sup> indicators with greatly  
982 improved fluorescence properties. *J Biol Chem* 260:3440–3450 . doi: 3838314

983 Han JH, Park SK, Lim CS, et al (2012) Simultaneous imaging of mitochondria and lysosomes  
984 by using two-photon fluorescent probes. *Chemistry* 18:15246–9 . doi:  
985 10.1002/chem.201203452

986 Hashimoto H, Isobe K, Suda A, et al (2010) Measurement of two-photon excitation spectra of  
987 fluorescent proteins with nonlinear Fourier-transform spectroscopy. *Appl Opt* 49:3323–  
988 9

989 Heinze KG, Koltermann A, Schwille P (2000) Simultaneous two-photon excitation of distinct  
990 labels for dual-color fluorescence crosscorrelation analysis. *Proc Natl Acad Sci U S A*

991 97:10377–82 . doi: 10.1073/pnas.180317197

992 Helassa N, Zhang X, Conte I, et al (2015) Fast-Response Calmodulin-Based Fluorescent  
993 Indicators Reveal Rapid Intracellular Calcium Dynamics. *Sci Rep* 5:15978 . doi:  
994 10.1038/srep15978

995 Helmchen F (2009) Two-Photon Functional Imaging of Neuronal Activity

996 Helmchen F, Denk W (2005) Deep tissue two-photon microscopy. *Nat Methods* 2:932–40 .  
997 doi: 10.1038/nmeth818

998 Heo CH, Kim KH, Kim HJ, et al (2013) A two-photon fluorescent probe for amyloid- $\beta$  plaques  
999 in living mice. *Chem Commun (Camb)* 49:1303–5 . doi: 10.1039/c2cc38570h

1000 Herron TJ, Lee P, Jalife J (2012) Optical imaging of voltage and calcium in cardiac cells &  
1001 tissues. *Circ Res* 110:609–623 . doi: 10.1161/CIRCRESAHA.111.247494

1002 Herz J, Siffrin V, Hauser AE, et al (2010) Expanding two-photon intravital microscopy to the  
1003 infrared by means of optical parametric oscillator. *Biophys J* 98:715–23 . doi:  
1004 10.1016/j.bpj.2009.10.035

1005 Hess GP, Lewis RW, Chen Y (2014) Caged neurotransmitters and other caged compounds:  
1006 design and application. *Cold Spring Harb Protoc* 2014:pdb.top084152 . doi:  
1007 10.1101/pdb.top084152

1008 Holtmaat A, Bonhoeffer T, Chow DK, et al (2009) Long-term, high-resolution imaging in the  
1009 mouse neocortex through a chronic cranial window. *Nat Protoc* 4:1128–44 . doi:  
1010 10.1038/nprot.2009.89

1011 Huber D, Petreanu L, Ghitani N, et al (2008) Sparse optical microstimulation in barrel cortex  
1012 drives learned behaviour in freely moving mice. *Nature* 451:61–64 . doi: nature06445  
1013 [pii] 10.1038/nature06445

1014 Im K-B, Kang M-S, Kim J, et al (2010) Two-photon spectral imaging with high temporal and

1015 spectral resolution. *Opt Express* 18:26905–14 . doi: 10.1364/OE.18.026905

1016 Ji N, Milkie DE, Betzig E (2010) Adaptive optics via pupil segmentation for high-resolution  
1017 imaging in biological tissues. *Nat Methods* 7:141–7 . doi: 10.1038/nmeth.1411

1018 Jin L, Han Z, Platisa J, et al (2012) Single action potentials and subthreshold electrical events  
1019 imaged in neurons with a fluorescent protein voltage probe. *Neuron* 75:779–85 . doi:  
1020 10.1016/j.neuron.2012.06.040

1021 Jung S, Aliberti J, Graemmel P, et al (2000) Analysis of fractalkine receptor CX(3)CR1 function  
1022 by targeted deletion and green fluorescent protein reporter gene insertion. *Mol Cell  
1023 Biol* 20:4106–14

1024 Junyent F, Kremer EJ (2015) CAV-2--why a canine virus is a neurobiologist's best friend. *Curr  
1025 Opin Pharmacol* 24:86–93 . doi: 10.1016/j.coph.2015.08.004

1026 Kamino K, Katoh Y, Komuro H, Sato K (1989) Multiple-site optical monitoring of neural  
1027 activity evoked by vagus nerve stimulation in the embryonic chick brain stem. *J Physiol*  
1028 409:263–83

1029 Kandel ER, Schwartz JH, Jessell TM (2000) *Principles of Neural Science*, 4th edn. McGraw-Hill

1030 Kao Y-T, Zhu X, Xu F, Min W (2012) Focal switching of photochromic fluorescent proteins  
1031 enables multiphoton microscopy with superior image contrast. *Biomed Opt Express*  
1032 3:1955–63 . doi: 10.1364/BOE.3.001955

1033 Karasawa S, Araki T, Nagai T, et al (2004) Cyan-emitting and orange-emitting fluorescent  
1034 proteins as a donor/acceptor pair for fluorescence resonance energy transfer. *Biochem  
1035 J* 381:307–12 . doi: 10.1042/BJ20040321

1036 Keikhosravi A, Bredfeldt JS, Sagar AK, Eliceiri KW (2014) Second-harmonic generation  
1037 imaging of cancer. *Methods Cell Biol* 123:531–46 . doi: 10.1016/B978-0-12-420138-  
1038 5.00028-8

1039 Khan M, Goldsmith CR, Huang Z, et al (2014) Two-photon imaging of Zn<sup>2+</sup> dynamics in mossy  
1040 fiber boutons of adult hippocampal slices. *Proc Natl Acad Sci U S A* 111:6786–91 . doi:  
1041 10.1073/pnas.1405154111

1042 Kim HJ, Han JH, Kim MK, et al (2010a) Dual-color imaging of sodium/calcium ion activities  
1043 with two-photon fluorescent probes. *Angew Chem Int Ed Engl* 49:6786–9 . doi:  
1044 10.1002/anie.201002907

1045 Kim HM, Choo H-J, Jung S-Y, et al (2007) A two-photon fluorescent probe for lipid raft  
1046 imaging: C-laurdan. *Chembiochem* 8:553–9 . doi: 10.1002/cbic.200700003

1047 Kim HM, Kim BR, An MJ, et al (2008a) Two-photon fluorescent probes for long-term imaging  
1048 of calcium waves in live tissue. *Chemistry* 14:2075–83 . doi: 10.1002/chem.200701453

1049 Kim HM, Seo MS, An MJ, et al (2008b) Two-photon fluorescent probes for intracellular free  
1050 zinc ions in living tissue. *Angew Chem Int Ed Engl* 47:5167–70 . doi:  
1051 10.1002/anie.200800929

1052 Kim JH, Lee S-R, Li L-H, et al (2011) High Cleavage Efficiency of a 2A Peptide Derived from  
1053 Porcine Teschovirus-1 in Human Cell Lines, Zebrafish and Mice. *PLoS One* 6:e18556 .  
1054 doi: 10.1371/journal.pone.0018556

1055 Kim MK, Lim CS, Hong JT, et al (2010b) Sodium-ion-selective two-photon fluorescent probe  
1056 for in vivo imaging. *Angew Chem Int Ed Engl* 49:364–7 . doi: 10.1002/anie.200904835

1057 Klunk WE, Bacskai BJ, Mathis CA, et al (2002) Imaging Abeta plaques in living transgenic mice  
1058 with multiphoton microscopy and methoxy-X04, a systemically administered Congo red  
1059 derivative. *J Neuropathol Exp Neurol* 61:797–805

1060 Kobat D, Durst ME, Nishimura N, et al (2009) Deep tissue multiphoton microscopy using  
1061 longer wavelength excitation. *Opt Express* 17:13354–13364 . doi:  
1062 10.1364/OE.17.013354

1063 Kobat D, Horton NG, Xu C (2011) In vivo two-photon microscopy to 1.6-mm depth in mouse  
1064 cortex. *J Biomed Opt* 16:106014 . doi: 10.1117/1.3646209

1065 König K (2000) Multiphoton microscopy in life sciences. *J Microsc* 200:83–104

1066 Kristianto J, Johnson MG, Zastrow RK, et al (2017) Spontaneous recombinase activity of Cre-  
1067 ERT2 in vivo. *Transgenic Res* 26:411–417 . doi: 10.1007/s11248-017-0018-1

1068 Kuhn B, Denk W, Bruno RM (2008) In vivo two-photon voltage-sensitive dye imaging reveals  
1069 top-down control of cortical layers 1 and 2 during wakefulness. *Proc Natl Acad Sci U S A*  
1070 105:7588–93 . doi: 10.1073/pnas.0802462105

1071 Kuhn B, Fromherz P, Denk W (2004) High sensitivity of Stark-shift voltage-sensing dyes by  
1072 one- or two-photon excitation near the red spectral edge. *Biophys J* 87:631–9 . doi:  
1073 10.1529/biophysj.104.040477

1074 Kuwashima N, Nishimura F, Eguchi J, et al (2005) Delivery of dendritic cells engineered to  
1075 secrete IFN- $\alpha$  into central nervous system tumors enhances the efficacy of  
1076 peripheral tumor cell vaccines: dependence on apoptotic pathways. *J Immunol*  
1077 175:2730–40

1078 Lane LA, Smith AM, Lian T, Nie S (2014) Compact and blinking-suppressed quantum dots for  
1079 single-particle tracking in live cells. *J Phys Chem B* 118:14140–7 . doi:  
1080 10.1021/jp5064325

1081 Langer J, Rose CR (2009) Synaptically induced sodium signals in hippocampal astrocytes in  
1082 situ. *J Physiol* 587:5859–77 . doi: 10.1113/jphysiol.2009.182279

1083 Larson DR, Zipfel WR, Williams RM, et al (2003) Water-soluble quantum dots for  
1084 multiphoton fluorescence imaging in vivo. *Science* 300:1434–6 . doi:  
1085 10.1126/science.1083780

1086 Lee JH, Lim CS, Tian YS, et al (2010) A two-photon fluorescent probe for thiols in live cells and

1087 tissues. *J Am Chem Soc* 132:1216–7 . doi: 10.1021/ja9090676

1088 Lee S, Lee JH, Park JH, et al (2016) In vivo 3D measurement of moxifloxacin and gatifloxacin  
1089 distributions in the mouse cornea using multiphoton microscopy. *Sci Rep* 6:25339 . doi:  
1090 10.1038/srep25339

1091 Li W, Fang B, Jin M, Tian Y (2017) Two-Photon Ratiometric Fluorescence Probe with  
1092 Enhanced Absorption Cross Section for Imaging and Biosensing of Zinc Ions in  
1093 Hippocampal Tissue and Zebrafish. *Anal Chem* 89:2553–2560 . doi:  
1094 10.1021/acs.analchem.6b04781

1095 Lim CS, Cho BR (2013) Two-photon probes for biomedical applications. *BMB Rep* 46:188–94 .  
1096 doi: 10.5483/BMBRep.2013.46.4.045

1097 Lim CS, Chung C, Kim HM, et al (2012) A two-photon turn-on probe for glucose uptake. *Chem*  
1098 *Commun (Camb)* 48:2122–4 . doi: 10.1039/c2cc16792a

1099 Lim CS, Kang MY, Han JH, et al (2011a) In vivo imaging of near-membrane calcium ions with  
1100 two-photon probes. *Chem Asian J* 6:2028–33 . doi: 10.1002/asia.201100156

1101 Lim CS, Kim HJ, Lee JH, et al (2011b) A two-photon turn-on probe for lipid rafts with  
1102 minimum internalization. *Chembiochem* 12:392–5 . doi: 10.1002/cbic.201000609

1103 Lippes J (2015) Quinacrine sterilization (QS): time for reconsideration. *Contraception* 92:91–  
1104 5 . doi: 10.1016/j.contraception.2015.06.005

1105 Liu H-W, Xu S, Wang P, et al (2016) An efficient two-photon fluorescent probe for monitoring  
1106 mitochondrial singlet oxygen in tissues during photodynamic therapy. *Chem Commun*  
1107 *(Camb)* 52:12330–12333 . doi: 10.1039/c6cc05880a

1108 Liu XF, Haas K (2011) Single-cell electroporation of *Xenopus* tadpole tectal neurons. *Cold*  
1109 *Spring Harb Protoc* 2011: . doi: 10.1101/pdb.prot065615

1110 Livet J, Weissman TA, Kang H, et al (2007) Transgenic strategies for combinatorial expression



1111 of fluorescent proteins in the nervous system. *Nature* 450:56–62 . doi:  
1112 10.1038/nature06293

1113 Loew LM, Lewis A (2015) Second Harmonic Imaging of Membrane Potential. *Adv Exp Med*  
1114 *Biol* 859:473–92 . doi: 10.1007/978-3-319-17641-3\_19

1115 Looger LL, Griesbeck O (2011) Genetically encoded neural activity indicators. *Curr Opin*  
1116 *Neurobiol* 22:18–23 . doi: 10.1016/j.conb.2011.10.024

1117 Lukomska J, Gryczynski I, Malicka J, et al (2006) One- and two-photon induced fluorescence  
1118 of Pacific Blue-labeled human serum albumin deposited on different core size silver  
1119 colloids. *Biopolymers* 81:249–55 . doi: 10.1002/bip.20407

1120 Lundby A, Mutoh H, Dimitrov D, et al (2008) Engineering of a genetically encodable  
1121 fluorescent voltage sensor exploiting fast Ci-VSP voltage-sensing movements. *PLoS One*  
1122 3:e2514 . doi: 10.1371/journal.pone.0002514

1123 Mahou P, Zimmerley M, Loulier K, et al (2012) Multicolor two-photon tissue imaging by  
1124 wavelength mixing. *Nat Methods* 9:815–8 . doi: 10.1038/nmeth.2098

1125 Manent J-B, Wang Y, Chang Y, et al (2009) Dcx reexpression reduces subcortical band  
1126 heterotopia and seizure threshold in an animal model of neuronal migration disorder.  
1127 *Nat Med* 15:84–90 . doi: 10.1038/nm.1897

1128 Mank M, Santos AF, Drenth S, et al (2008) A genetically encoded calcium indicator for  
1129 chronic in vivo two-photon imaging. *Nat Methods* 5:805–811 . doi:  
1130 10.1038/nmeth.1243

1131 Marchuk K, Guo Y, Sun W, et al (2012) High-precision tracking with non-blinking quantum  
1132 dots resolves nanoscale vertical displacement. *J Am Chem Soc* 134:6108–11 . doi:  
1133 10.1021/ja301332t

1134 Marshall JD, Li JZ, Zhang Y, et al (2016) Cell-Type-Specific Optical Recording of Membrane

1135 Voltage Dynamics in Freely Moving Mice. *Cell* 167:1650–1662.e15 . doi:  
1136 10.1016/j.cell.2016.11.021

1137 Marvin JS, Borghuis BG, Tian L, et al (2013) An optimized fluorescent probe for visualizing  
1138 glutamate neurotransmission. *Nat Methods* 10:162–70 . doi: 10.1038/nmeth.2333

1139 Masanta G, Lim CS, Kim HJ, et al (2011) A mitochondrial-targeted two-photon probe for zinc  
1140 ion. *J Am Chem Soc* 133:5698–700 . doi: 10.1021/ja200444t

1141 Mashinchian O, Johari-Ahar M, Ghaemi B, et al (2014) Impacts of quantum dots in molecular  
1142 detection and bioimaging of cancer. *Bioimpacts* 4:149–66 . doi: 10.15171/bi.2014.008

1143 Matsuda T, Cepko CL (2004) Electroporation and RNA interference in the rodent retina in  
1144 vivo and in vitro. *Proc Natl Acad Sci U S A* 101:16–22

1145 Matsuda T, Cepko CL (2007) Controlled expression of transgenes introduced by in vivo  
1146 electroporation. *Proc Natl Acad Sci U S A* 104:1027–32 . doi: 10.1073/pnas.0610155104

1147 Merzlyak EM, Goedhart J, Shcherbo D, et al (2007) Bright monomeric red fluorescent protein  
1148 with an extended fluorescence lifetime. *Nat Methods* 4:555–7 . doi:  
1149 10.1038/nmeth1062

1150 Mettra B, Appaix F, Olesiak-Banska J, et al (2016) A Fluorescent Polymer Probe with High  
1151 Selectivity toward Vascular Endothelial Cells for and beyond Noninvasive Two-Photon  
1152 Intravital Imaging of Brain Vasculature. *ACS Appl Mater Interfaces* 8:17047–59 . doi:  
1153 10.1021/acsami.6b02936

1154 Miao F, Zhang W, Sun Y, et al (2014) Novel fluorescent probes for highly selective two-  
1155 photon imaging of mitochondria in living cells. *Biosens Bioelectron* 55:423–9 . doi:  
1156 10.1016/j.bios.2013.12.044

1157 Miller MJ, Wei SH, Parker I, Cahalan MD (2002) Two-photon imaging of lymphocyte motility  
1158 and antigen response in intact lymph node. *Science* 296:1869–73 . doi:

1159           10.1126/science.1070051

1160 Miyawaki a, Llopis J, Heim R, et al (1997) Fluorescent indicators for Ca<sup>2+</sup> based on green  
1161           fluorescent proteins and calmodulin. *Nature* 388:882–887 . doi: 10.1038/42264

1162 Miyazaki K, Ross WN (2015) Simultaneous Sodium and Calcium Imaging from Dendrites and  
1163           Axons. *eNeuro* 2:1–10 . doi: 10.1523/ENEURO.0092-15.2015

1164 Mohan PS, Lim CS, Tian YS, et al (2009) A two-photon fluorescent probe for near-membrane  
1165           calcium ions in live cells and tissues. *Chem Commun (Camb)* 5365–7 . doi:  
1166           10.1039/b911337a

1167 Mojzisoava H, Vermot J (2011) When multiphoton microscopy sees near infrared. *Curr Opin*  
1168           *Genet Dev* 21:549–57 . doi: 10.1016/j.gde.2011.08.004

1169 Momose-Sato Y, Sato K, Kamino K (2001) Optical approaches to embryonic development of  
1170           neural functions in the brainstem. *Prog Neurobiol* 63:151–97

1171 Morelli AE, Larregina AT, Shufesky WJ, et al (2003) Internalization of circulating apoptotic  
1172           cells by splenic marginal zone dendritic cells: dependence on complement receptors  
1173           and effect on cytokine production. *Blood* 101:611–20 . doi: 10.1182/blood-2002-06-  
1174           1769

1175 Moritomo H, Yamada K, Kojima Y, et al (2014) A biphenyl type two-photon fluorescence  
1176           probe for monitoring the mitochondrial membrane potential. *Cell Struct Funct* 39:125–  
1177           33 . doi: 10.1247/csf.14006

1178 Mossakowski AA, Pohlan J, Bremer D, et al (2015) Tracking CNS and systemic sources of  
1179           oxidative stress during the course of chronic neuroinflammation. *Acta Neuropathol*  
1180           130:799–814 . doi: 10.1007/s00401-015-1497-x

1181 Mostany R, Miquelajauregui A, Shtrahman M, Portera-Cailliau C (2015) Two-photon  
1182           excitation microscopy and its applications in neuroscience. *Methods Mol Biol* 1251:25–

1183 42 . doi: 10.1007/978-1-4939-2080-8\_2

1184 Murphy TH, Li P, Betts K, Liu R (2008) Two-photon imaging of stroke onset in vivo reveals  
1185 that NMDA-receptor independent ischemic depolarization is the major cause of rapid  
1186 reversible damage to dendrites and spines. *J Neurosci* 28:1756–72 . doi:  
1187 10.1523/JNEUROSCI.5128-07.2008

1188 Mütze J, Iyer V, Macklin JJ, et al (2012) Excitation spectra and brightness optimization of  
1189 two-photon excited probes. *Biophys J* 102:934–44 . doi: 10.1016/j.bpj.2011.12.056

1190 Myoga MH, Beierlein M, Regehr WG (2009) Somatic spikes regulate dendritic signaling in  
1191 small neurons in the absence of backpropagating action potentials. *J Neurosci* 29:7803–  
1192 14 . doi: 10.1523/JNEUROSCI.0030-09.2009

1193 Nagai T, Ibata K, Park ES, et al (2002) A variant of yellow fluorescent protein with fast and  
1194 efficient maturation for cell-biological applications. *Nat Biotechnol* 20:87–90 . doi:  
1195 10.1038/nbt0102-87

1196 Nagai T, Yamada S, Tominaga T, et al (2004) Expanded dynamic range of fluorescent  
1197 indicators for Ca(2+) by circularly permuted yellow fluorescent proteins. *Proc Natl Acad*  
1198 *Sci U S A* 101:10554–10559 . doi: 10.1073/pnas.0400417101

1199 Nakahira E, Kagawa T, Shimizu T, et al (2006) Direct evidence that ventral forebrain cells  
1200 migrate to the cortex and contribute to the generation of cortical myelinating  
1201 oligodendrocytes. *Dev Biol* 291:123–131 . doi: 10.1016/j.ydbio.2005.12.010

1202 Nakai J, Ohkura M, Imoto K (2001) A high signal-to-noise Ca(2+) probe composed of a single  
1203 green fluorescent protein. *Nat Biotechnol* 19:137–41 . doi: 10.1038/84397

1204 Nassi JJ, Cepko CL, Born RT, Beier KT (2015) Neuroanatomy goes viral! *Front Neuroanat* 9:80  
1205 . doi: 10.3389/fnana.2015.00080

1206 Navarro-Quiroga I, Chittajallu R, Gallo V, Haydar TF (2007) Long-Term, Selective Gene

1207 Expression in Developing and Adult Hippocampal Pyramidal Neurons Using Focal In  
1208 Utero Electroporation. *J Neurosci* 27:

1209 Neher R, Neher E (2004) Optimizing imaging parameters for the separation of multiple labels  
1210 in a fluorescence image. *J Microsc* 213:46–62

1211 Nemoto T, Kawakami R, Hibi T, et al (2015) Two-photon excitation fluorescence microscopy  
1212 and its application in functional connectomics. *Reprod Syst Sex Disord* 64:9–15 . doi:  
1213 10.1093/jmicro/dfu110

1214 Neu TR, Kuhlicke U, Lawrence JR (2002) Assessment of fluorochromes for two-photon laser  
1215 scanning microscopy of biofilms. *Appl Environ Microbiol* 68:901–9

1216 Nimmerjahn A, Kirchhoff F, Kerr JND, Helmchen F (2004) Sulforhodamine 101 as a specific  
1217 marker of astroglia in the neocortex in vivo. *Nat Methods* 1:31–7 . doi:  
1218 10.1038/nmeth706

1219 Packer AM, Russell LE, Dagleish HWP, Häusser M (2015) Simultaneous all-optical  
1220 manipulation and recording of neural circuit activity with cellular resolution in vivo. *Nat*  
1221 *Methods* 12:140–6 . doi: 10.1038/nmeth.3217

1222 Pak YL, Swamy KMK, Yoon J (2015) Recent Progress in Fluorescent Imaging Probes. *Sensors*  
1223 (Basel) 15:24374–96 . doi: 10.3390/s150924374

1224 Papagiakoumou E, Bègue A, Leshem B, et al (2013) Functional patterned multiphoton  
1225 excitation deep inside scattering tissue. *Nat Photonics* 7:274–278 . doi:  
1226 10.1038/nphoton.2013.9

1227 Park HJ, Lim CS, Kim ES, et al (2012) Measurement of pH values in human tissues by two-  
1228 photon microscopy. *Angew Chem Int Ed Engl* 51:2673–6 . doi: 10.1002/anie.201109052

1229 Petersen CCH, Grinvald A, Sakmann B (2003) Spatiotemporal dynamics of sensory responses  
1230 in layer 2/3 of rat barrel cortex measured in vivo by voltage-sensitive dye imaging

1231 combined with whole-cell voltage recordings and neuron reconstructions. *J Neurosci*  
1232 23:1298–309

1233 Petrat F, Rauen U, de Groot H (1999) Determination of the chelatable iron pool of isolated  
1234 rat hepatocytes by digital fluorescence microscopy using the fluorescent probe, phen  
1235 green SK. *Hepatology* 29:1171–9 . doi: 10.1002/hep.510290435

1236 Piatkevich KD, Hult J, Subach OM, et al (2010) Monomeric red fluorescent proteins with a  
1237 large Stokes shift. *Proc Natl Acad Sci U S A* 107:5369–74 . doi:  
1238 10.1073/pnas.0914365107

1239 Podgorski K, Terpetschnig E, Klochko OP, et al (2012) Ultra-bright and -stable red and near-  
1240 infrared squaraine fluorophores for in vivo two-photon imaging. *PLoS One* 7:e51980 .  
1241 doi: 10.1371/journal.pone.0051980

1242 Pond SJK, Rumi M, Levin MD, et al (2002) One- and Two-Photon Spectroscopy of  
1243 Donor–Acceptor–Donor Distyrylbenzene Derivatives: Effect of Cyano Substitution and  
1244 Distortion from Planarity. *J Phys Chem A* 106:11470–11480 . doi: 10.1021/jp0267104

1245 Porrero C, Rubio-Garrido P, Avendaño C, Clascá F (2010) Mapping of fluorescent protein-  
1246 expressing neurons and axon pathways in adult and developing Thy1-eYFP-H transgenic  
1247 mice. *Brain Res* 1345:59–72 . doi: 10.1016/j.brainres.2010.05.061

1248 Poulet JFA, Petersen CCH (2008) Internal brain state regulates membrane potential  
1249 synchrony in barrel cortex of behaving mice. *Nature* 454:881–5 . doi:  
1250 10.1038/nature07150

1251 Powell SK, Khan N, Parker CL, et al (2016) Characterization of a novel adeno-associated viral  
1252 vector with preferential oligodendrocyte tropism. *Gene Ther* 23:807–814 . doi:  
1253 10.1038/gt.2016.62

1254 Prakash R, Yizhar O, Grewe B, et al (2012) Two-photon optogenetic toolbox for fast

1255 inhibition, excitation and bistable modulation. *Nat Methods* 9:1171–9 . doi:  
1256 10.1038/nmeth.2215

1257 Radbruch H, Bremer D, Guenther R, et al (2016) Ongoing Oxidative Stress Causes Subclinical  
1258 Neuronal Dysfunction in the Recovery Phase of EAE. *Front Immunol* 7:92 . doi:  
1259 10.3389/fimmu.2016.00092

1260 Rakhymzhan A, Leben R, Zimmermann H, et al (2017) Synergistic Strategy for Multicolor  
1261 Two-photon Microscopy: Application to the Analysis of Germinal Center Reactions In  
1262 Vivo. *Sci Rep* 7:7101 . doi: 10.1038/s41598-017-07165-0

1263 Rakic P (1974) Neurons in Rhesus Monkey Visual Cortex: Systematic Relation between Time  
1264 of Origin and Eventual Disposition. *Science* (80- ) 183:

1265 Rama S, Vetrivel L, Semyanov A (2010) Second-harmonic generation voltage imaging at  
1266 subcellular resolution in rat hippocampal slices. *J Biophotonics* 3:784–90 . doi:  
1267 10.1002/jbio.201000073

1268 Reeves AMB, Shigetomi E, Khakh BS (2011) Bulk loading of calcium indicator dyes to study  
1269 astrocyte physiology: key limitations and improvements using morphological maps. *J*  
1270 *Neurosci* 31:9353–8 . doi: 10.1523/JNEUROSCI.0127-11.2011

1271 Resendez SL, Jennings JH, Ung RL, et al (2016) Visualization of cortical, subcortical and deep  
1272 brain neural circuit dynamics during naturalistic mammalian behavior with head-  
1273 mounted microscopes and chronically implanted lenses. *Nat Protoc* 11:566–97 . doi:  
1274 10.1038/nprot.2016.021

1275 Ricard C, Debarbieux FC (2014) Six-color intravital two-photon imaging of brain tumors and  
1276 their dynamic microenvironment. *Front Cell Neurosci* 8:57 . doi:  
1277 10.3389/fncel.2014.00057

1278 Ricard C, Fernández M, Gastaldo J, et al (2009) Short-term effects of synchrotron irradiation

1279 on vasculature and tissue in healthy mouse brain. *J Synchrotron Radiat* 16:477–83 . doi:  
1280 10.1107/S0909049509015428

1281 Ricard C, Fernandez M, Requardt H, et al (2013a) Synergistic effect of cisplatin and  
1282 synchrotron irradiation on F98 gliomas growing in nude mice. *J Synchrotron Radiat*  
1283 20:777–84 . doi: 10.1107/S0909049513016567

1284 Ricard C, Lamasse L, Jaouen A, et al (2016a) Combination of an optical parametric oscillator  
1285 and quantum-dots 655 to improve imaging depth of vasculature by intravital multicolor  
1286 two-photon microscopy. *Biomed Opt Express* 7:2362–72 . doi: 10.1364/BOE.7.002362

1287 Ricard C, Stanchi F, Rodriguez T, et al (2013b) Dynamic quantitative intravital imaging of  
1288 glioblastoma progression reveals a lack of correlation between tumor growth and blood  
1289 vessel density. *PLoS One* 8:e72655 . doi: 10.1371/journal.pone.0072655

1290 Ricard C, Tchoghandjian A, Luche H, et al (2016b) Phenotypic dynamics of microglial and  
1291 monocyte-derived cells in glioblastoma-bearing mice. *Sci Rep* 6:26381 . doi:  
1292 10.1038/srep26381

1293 Ricard C, Vacca B, Weber P (2012) Three-dimensional imaging of small intestine morphology  
1294 using non-linear optical microscopy and endogenous signals. *J Anat* 221:279–83 . doi:  
1295 10.1111/j.1469-7580.2012.01529.x

1296 Ricard C, Vial J-C, Douady J, van der Sanden B (2007) In vivo imaging of elastic fibers using  
1297 sulforhodamine B. *J Biomed Opt* 12:64017 . doi: 10.1117/1.2821421

1298 Rickgauer JP, Deisseroth K, Tank DW (2014) Simultaneous cellular-resolution optical  
1299 perturbation and imaging of place cell firing fields. *Nat Neurosci* 17:1816–24 . doi:  
1300 10.1038/nn.3866

1301 Rizzo MA, Springer G, Segawa K, et al (2006) Optimization of pairings and detection  
1302 conditions for measurement of FRET between cyan and yellow fluorescent proteins.



1303           Microsc Microanal 12:238–54 . doi: 10.1017/S1431927606060235

1304 Roder P, Hille C (2014) ANG-2 for quantitative Na(+) determination in living cells by time-  
1305           resolved fluorescence microscopy. Photochem Photobiol Sci 13:1699–710 . doi:  
1306           10.1039/c4pp00061g

1307 Romanelli E, Sorbara CD, Nikić I, et al (2013) Cellular, subcellular and functional in vivo  
1308           labeling of the spinal cord using vital dyes. Nat Protoc 8:481–90 . doi:  
1309           10.1038/nprot.2013.022

1310 Ruthazer ES, Cline HT (2002) Multiphoton Imaging of Neurons in Living Tissue: Acquisition  
1311           and Analysis of Time-Lapse Morphological Data. Real-Time Imaging 8:175–188 . doi:  
1312           10.1006/rtim.2002.0284

1313 Sabatini BL, Oertner TG, Svoboda K (2002) The life cycle of Ca(2+) ions in dendritic spines.  
1314           Neuron 33:439–52

1315 Sadowski B, Kita H, Grzybowski M, et al (2017)  $\pi$ -Expanded Dipyrrolonaphthyridinediones  
1316           with Large Two-Photon Absorption Cross-Section Values. J Org Chem. doi:  
1317           10.1021/acs.joc.7b00831

1318 Saito T, Nakatsuji N (2001) Efficient gene transfer into the embryonic mouse brain using in  
1319           vivo electroporation. Dev Biol 240:237–246

1320 Santisakultarm TP, Kersbergen CJ, Bandy DK, et al (2016) Two-photon imaging of cerebral  
1321           hemodynamics and neural activity in awake and anesthetized marmosets. J Neurosci  
1322           Methods 271:55–64 . doi: 10.1016/j.jneumeth.2016.07.003

1323 Saria A, Lundberg JM (1983) Evans blue fluorescence: quantitative and morphological  
1324           evaluation of vascular permeability in animal tissues. J Neurosci Methods 8:41–9

1325 Sarkar P, Koushik S V, Vogel SS, et al (2009) Photophysical properties of Cerulean and Venus  
1326           fluorescent proteins. J Biomed Opt 14:34047 . doi: 10.1117/1.3156842

1327 Schaffer CB, Friedman B, Nishimura N, et al (2006) Two-photon imaging of cortical surface  
1328 microvessels reveals a robust redistribution in blood flow after vascular occlusion. *PLoS*  
1329 *Biol* 4:e22 . doi: 10.1371/journal.pbio.0040022

1330 Schwarze T, Riemer J, Eidner S, Holdt H-J (2015) A Highly K(+)-Selective Two-Photon  
1331 Fluorescent Probe. *Chemistry* 21:11306–10 . doi: 10.1002/chem.201501473

1332 Senatorov V V., Stys PK, Hu B (2000) Regulation of Na<sup>+</sup>,K<sup>+</sup>-ATPase by persistent sodium  
1333 accumulation in adult rat thalamic neurones. *J Physiol* 525 Pt 2:343–53 . doi:  
1334 10.1111/j.1469-7793.2000.00343.x

1335 Seo EW, Han JH, Heo CH, et al (2012) A small-molecule two-photon probe for nitric oxide in  
1336 living tissues. *Chemistry* 18:12388–94 . doi: 10.1002/chem.201201197

1337 Shaner NC, Campbell RE, Steinbach PA, et al (2004) Improved monomeric red, orange and  
1338 yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat*  
1339 *Biotechnol* 22:1567–72 . doi: 10.1038/nbt1037

1340 Shaner NC, Lin MZ, McKeown MR, et al (2008) Improving the photostability of bright  
1341 monomeric orange and red fluorescent proteins. *Nat Methods* 5:545–51 . doi:  
1342 10.1038/nmeth.1209

1343 Shaner NC, Steinbach PA, Tsien RY (2005) A guide to choosing fluorescent proteins. *Nat*  
1344 *Methods* 2:905–9 . doi: 10.1038/nmeth819

1345 Shcherbo D, Merzlyak EM, Chepurnykh T V, et al (2007) Bright far-red fluorescent protein for  
1346 whole-body imaging. *Nat Methods* 4:741–6 . doi: 10.1038/nmeth1083

1347 Shcherbo D, Murphy CS, Ermakova G V, et al (2009) Far-red fluorescent tags for protein  
1348 imaging in living tissues. *Biochem J* 418:567–74 . doi: 10.1042/BJ20081949

1349 Shen Y, Dana H, Abdelfattah AS, et al (2018) A genetically encoded Ca<sup>2+</sup>indicator based on  
1350 circularly permuted sea anemone red fluorescent protein eqFP578. *BMC Biol* 16:9 .

1351 doi: 10.1186/s12915-018-0480-0

1352 Shen Z, Lu Z, Chhatbar PY, et al (2012) An artery-specific fluorescent dye for studying  
1353 neurovascular coupling. *Nat Methods* 9:273–6 . doi: 10.1038/nmeth.1857

1354 Shi X, Li M, Zhao W, et al (2015) Spectral imaging superlocalization microscopy for quantum  
1355 dots. *Sensors Actuators B Chem* 207:308–312 . doi: 10.1016/j.snb.2014.10.077

1356 Shi X, Xie Z, Song Y, et al (2012) Superlocalization spectral imaging microscopy of a multicolor  
1357 quantum dot complex. *Anal Chem* 84:1504–9 . doi: 10.1021/ac202784h

1358 Shigetomi E, Bushong EA, Haustein MD, et al (2013) Imaging calcium microdomains within  
1359 entire astrocyte territories and endfeet with GCaMPs expressed using adeno-associated  
1360 viruses. *J Gen Physiol* 141:633–47 . doi: 10.1085/jgp.201210949

1361 Shigetomi E, Patel S, Khakh BS (2016) Probing the Complexities of Astrocyte Calcium  
1362 Signaling. *Trends Cell Biol* 26:300–12 . doi: 10.1016/j.tcb.2016.01.003

1363 Shoham D, Glaser DE, Arieli A, et al (1999) Imaging cortical dynamics at high spatial and  
1364 temporal resolution with novel blue voltage-sensitive dyes. *Neuron* 24:791–802

1365 Siegel MS, Isacoff EY (1997) A genetically encoded optical probe of membrane voltage.  
1366 *Neuron* 19:735–41

1367 Singh H, Lee HW, Heo CH, et al (2015) A Golgi-localized two-photon probe for imaging zinc  
1368 ions. *Chem Commun (Camb)* 51:12099–102 . doi: 10.1039/c5cc03884g

1369 Smith PG, Baldacchini T, Carter J, Zadoyan R (2012) TWO-PHOTON  
1370 MICROSCOPY/MULTIMODAL IMAGING: Femtosecond laser developments advance two-  
1371 photon imaging. In: *BioOptics World*.  
1372 [http://www.bioopticsworld.com/articles/print/volume-5/issue-](http://www.bioopticsworld.com/articles/print/volume-5/issue-04/features/femtosecond-laser-developments-advance-two-photon-imaging.html)  
1373 [04/features/femtosecond-laser-developments-advance-two-photon-imaging.html](http://www.bioopticsworld.com/articles/print/volume-5/issue-04/features/femtosecond-laser-developments-advance-two-photon-imaging.html).  
1374 Accessed 1 Jan 2017

1375 So PT, Dong CY, Masters BR, Berland KM (2000) Two-photon excitation fluorescence  
1376 microscopy. *Annu Rev Biomed Eng* 2:399–429 . doi: 10.1146/annurev.bioeng.2.1.399  
1377 Son JH, Lim CS, Han JH, et al (2011) Two-photon LysoTrackers for in vivo imaging. *J Org Chem*  
1378 76:8113–6 . doi: 10.1021/jo201504h  
1379 Srinivasan R, Huang BS, Venugopal S, et al (2015) Ca<sup>2+</sup> signaling in astrocytes from *Ip3r2*<sup>-/-</sup>  
1380 mice in brain slices and during startle responses in vivo. doi: 10.1038/nn.4001  
1381 St-Pierre F, Marshall JD, Yang Y, et al (2014) High-fidelity optical reporting of neuronal  
1382 electrical activity with an ultrafast fluorescent voltage sensor. *Nat Neurosci* 17:884–9 .  
1383 doi: 10.1038/nn.3709  
1384 Stringari C, Abdeladim L, Malkinson G, et al (2017) Multicolor two-photon imaging of  
1385 endogenous fluorophores in living tissues by wavelength mixing. *Sci Rep* 7:3792 . doi:  
1386 10.1038/s41598-017-03359-8  
1387 Stringari C, Nourse JL, Flanagan LA, Gratton E (2012) Phasor fluorescence lifetime microscopy  
1388 of free and protein-bound NADH reveals neural stem cell differentiation potential. *PLoS*  
1389 *One* 7:e48014 . doi: 10.1371/journal.pone.0048014  
1390 Svoboda K, Yasuda R (2006) Principles of two-photon excitation microscopy and its  
1391 applications to neuroscience. *Neuron* 50:823–39 . doi: 10.1016/j.neuron.2006.05.019  
1392 Swirski FK, Berger CR, Figueiredo J-L, et al (2007) A near-infrared cell tracker reagent for  
1393 multiscope in vivo imaging and quantification of leukocyte immune responses. *PLoS*  
1394 *One* 2:e1075 . doi: 10.1371/journal.pone.0001075  
1395 Tabata H, Nakajima K (2001) Efficient in utero gene transfer system to the developing mouse  
1396 brain using electroporation: visualization of neuronal migration in the developing  
1397 cortex. *Neuroscience* 103:865–872 . doi: 10.1016/S0306-4522(01)00016-1  
1398 Takahashi M, Sato K, Nomura T, Osumi N (2002) Manipulating gene expressions by

1399 electroporation in the developing brain of mammalian embryos. *Differentiation*  
1400 70:155–162 . doi: 10.1046/j.1432-0436.2002.700405.x

1401 Takaki M, Goto K, Kawahara I, Nabekura J (2015) Activation of 5-HT4 receptors facilitates  
1402 neurogenesis of injured enteric neurons at an anastomosis in the lower gut. *J Smooth*  
1403 *Muscle Res* 51:82–94 . doi: 10.1540/jsmr.51.82

1404 Takiguchi-Hayashi K, Sekiguchi M, Ashigaki S, et al (2004) Generation of reelin-positive  
1405 marginal zone cells from the caudomedial wall of telencephalic vesicles. *J Neurosci*  
1406 24:2286–2295

1407 Tallini YN, Ohkura M, Choi B-R, et al (2006) Imaging cellular signals in the heart in vivo:  
1408 Cardiac expression of the high-signal Ca<sup>2+</sup> indicator GCaMP2. *Proc Natl Acad Sci U S A*  
1409 103:4753–8 . doi: 10.1073/pnas.0509378103

1410 Tervo DGR, Hwang B-Y, Viswanathan S, et al (2016) A Designer AAV Variant Permits Efficient  
1411 Retrograde Access to Projection Neurons. *Neuron* 92:372–382 . doi:  
1412 10.1016/j.neuron.2016.09.021

1413 Thaler C, Vogel SS (2006) Quantitative linear unmixing of CFP and YFP from spectral images  
1414 acquired with two-photon excitation. *Cytometry A* 69:904–11 . doi:  
1415 10.1002/cyto.a.20267

1416 Tischbirek C, Birkner A, Jia H, et al (2015) Deep two-photon brain imaging with a red-shifted  
1417 fluorometric Ca<sup>2+</sup> indicator. *Proc Natl Acad Sci U S A* 112:11377–82 . doi:  
1418 10.1073/pnas.1514209112

1419 Tozer GM, Ameer-Beg SM, Baker J, et al (2005) Intravital imaging of tumour vascular  
1420 networks using multi-photon fluorescence microscopy. *Adv Drug Deliv Rev* 57:135–52 .  
1421 doi: 10.1016/j.addr.2004.07.015

1422 Trägårdh J, Robb G, Amor R, et al (2015) Exploration of the two-photon excitation spectrum

1423 of fluorescent dyes at wavelengths below the range of the Ti:Sapphire laser. *J Microsc*  
1424 259:210–8 . doi: 10.1111/jmi.12255

1425 Tran CHT, Gordon GR (2015) Astrocyte and microvascular imaging in awake animals using  
1426 two-photon microscopy. *Microcirculation* 22:219–27 . doi: 10.1111/micc.12188

1427 Tsien RY, Rink TJ, Poenie M (1985) Measurement of cytosolic free Ca<sup>2+</sup> in individual small  
1428 cells using fluorescence microscopy with dual excitation wavelengths. *Cell Calcium*  
1429 6:145–157 . doi: 10.1016/0143-4160(85)90041-7

1430 Vadakkan TJ, Culver JC, Gao L, et al (2009) Peak Multiphoton Excitation of mCherry Using an  
1431 Optical Parametric Oscillator (OPO). *J Fluoresc* 19:1103–1109 . doi: 10.1007/s10895-  
1432 009-0510-y

1433 Velasco MGM, Allgeyer ES, Yuan P, et al (2015) Absolute two-photon excitation spectra of  
1434 red and far-red fluorescent probes. *Opt Lett* 40:4915–8

1435 Vérant P, Ricard C, Serduc R, et al (2013) In vivo staining of neocortical astrocytes via the  
1436 cerebral microcirculation using sulforhodamine B. *J Biomed Opt* 13:64028 . doi:  
1437 10.1117/1.3041163

1438 Wallace DJ (1989) The use of quinacrine (Atabrine) in rheumatic diseases: a reexamination.  
1439 *Semin Arthritis Rheum* 18:282–96

1440 Wang C, Mei L (2013) In utero electroporation in mice. *Methods Mol Biol* 1018:151–63 . doi:  
1441 10.1007/978-1-62703-444-9\_15

1442 Wang C, Yeh AT (2012) Two-photon excited fluorescence enhancement with broadband  
1443 versus tunable femtosecond laser pulse excitation. *J Biomed Opt* 17:25003 . doi:  
1444 10.1117/1.JBO.17.2.025003

1445 Wang F, Qi LS (2016) Applications of CRISPR Genome Engineering in Cell Biology. *Trends Cell*  
1446 *Biol* 26:875–888 . doi: 10.1016/j.tcb.2016.08.004

1447 Wang K, Sun W, Richie CT, et al (2015) Direct wavefront sensing for high-resolution in vivo  
1448 imaging in scattering tissue. *Nat Commun* 6:7276 . doi: 10.1038/ncomms8276

1449 Weigelin B, Bakker G-J, Friedl P (2016) Third harmonic generation microscopy of cells and  
1450 tissue organization. *J Cell Sci* 129:245–55 . doi: 10.1242/jcs.152272

1451 Weissman TA, Pan YA (2015) Brainbow: new resources and emerging biological applications  
1452 for multicolor genetic labeling and analysis. *Genetics* 199:293–306 . doi:  
1453 10.1534/genetics.114.172510

1454 Wickersham IR, Lyon DC, Barnard RJO, et al (2007) Monosynaptic restriction of transsynaptic  
1455 tracing from single, genetically targeted neurons. *Neuron* 53:639–47 . doi:  
1456 10.1016/j.neuron.2007.01.033

1457 Williams RM, Zipfel WR, Webb WW (2005) Interpreting second-harmonic generation images  
1458 of collagen I fibrils. *Biophys J* 88:1377–86 . doi: 10.1529/biophysj.104.047308

1459 Witte S, Negrean A, Lodder JC, et al (2011) Label-free live brain imaging and targeted  
1460 patching with third-harmonic generation microscopy. *Proc Natl Acad Sci U S A*  
1461 108:5970–5 . doi: 10.1073/pnas.1018743108

1462 Woodard LE, Wilson MH (2015) piggyBac-ing models and new therapeutic strategies. *Trends*  
1463 *Biotechnol* 33:525–33 . doi: 10.1016/j.tibtech.2015.06.009

1464 Xu C, Webb WW (1996) Measurement of two-photon excitation cross sections of molecular  
1465 fluorophores with data from 690 to 1050 nm. *J Opt Soc Am B* 13:481 . doi:  
1466 10.1364/JOSAB.13.000481

1467 Xu Y, Zou P, Cohen AE (2017) Voltage imaging with genetically encoded indicators. *Curr Opin*  
1468 *Chem Biol* 39:1–10 . doi: 10.1016/j.cbpa.2017.04.005

1469 Yan P, Acker CD, Zhou W-L, et al (2012) Palette of fluorinated voltage-sensitive hemicyanine  
1470 dyes. *Proc Natl Acad Sci U S A* 109:20443–8 . doi: 10.1073/pnas.1214850109

1471 Yang G, Pan F, Parkhurst CN, et al (2010) Thinned-skull cranial window technique for long-  
1472 term imaging of the cortex in live mice. *Nat Protoc* 5:201–8 . doi:  
1473 10.1038/nprot.2009.222

1474 Yasuda R, Harvey CD, Zhong H, et al (2006) Supersensitive Ras activation in dendrites and  
1475 spines revealed by two-photon fluorescence lifetime imaging. *Nat Neurosci* 9:283–291 .  
1476 doi: 10.1038/nn1635

1477 Yasuda R, Nimchinsky EA, Scheuss V, et al (2004) Imaging calcium concentration dynamics in  
1478 small neuronal compartments. *Sci STKE* 2004:pl5 . doi: 10.1126/stke.2192004pl5

1479 Yi KD, Covey DF, Simpkins JW (2009) Mechanism of okadaic acid-induced neuronal death and  
1480 the effect of estrogens. *J Neurochem* 108:732–40 . doi: 10.1111/j.1471-  
1481 4159.2008.05805.x

1482 Yin H, Zhang B, Yu H, et al (2015) Two-photon fluorescent probes for biological Mg(2+)  
1483 detection based on 7-substituted coumarin. *J Org Chem* 80:4306–12 . doi:  
1484 10.1021/jo502775t

1485 Yu H, Senarathna J, Tyler BM, et al (2015) Miniaturized optical neuroimaging in unrestrained  
1486 animals. *Neuroimage* 113:397–406 . doi: 10.1016/j.neuroimage.2015.02.070

1487 Yuste R, MacLean J, Vogelstein J, Paninski L (2011) Imaging Action Potentials with Calcium  
1488 Indicators. *Cold Spring Harb Protoc* 2011:pdb.prot5650-prot5650 . doi:  
1489 10.1101/pdb.prot5650

1490 Zapata-Hommer O, Griesbeck O (2003) Efficiently folding and circularly permuted variants of  
1491 the Sapphire mutant of GFP. *BMC Biotechnol* 3:5 . doi: 10.1186/1472-6750-3-5

1492 Zariwala H a, Borghuis BG, Hoogland TM, et al (2012) A Cre-dependent GCaMP3 reporter  
1493 mouse for neuronal imaging in vivo. *J Neurosci* 32:3131–41 . doi:  
1494 10.1523/JNEUROSCI.4469-11.2012



1495 Zehentbauer FM, Moretto C, Stephen R, et al (2014) Fluorescence spectroscopy of  
1496 Rhodamine 6G: concentration and solvent effects. *Spectrochim Acta A Mol Biomol*  
1497 *Spectrosc* 121:147–51 . doi: 10.1016/j.saa.2013.10.062

1498 Zhang H, Liu J, Wang L, et al (2018) Amino-Si-rhodamines: A new class of two-photon  
1499 fluorescent dyes with intrinsic targeting ability for lysosomes. *Biomaterials* 158:10–22 .  
1500 doi: 10.1016/j.biomaterials.2017.12.013

1501 Zhou W-L, Yan P, Wuskell JP, et al (2007) Intracellular long-wavelength voltage-sensitive dyes  
1502 for studying the dynamics of action potentials in axons and thin dendrites. *J Neurosci*  
1503 *Methods* 164:225–39 . doi: 10.1016/j.jneumeth.2007.05.002

1504 Zhu H, Derksen R, Krause C, et al (2005) Fluorescent Intensity of Dye Solutions under  
1505 Different pH Conditions. *J ASTM Int* 2:12926 . doi: 10.1520/JAI12926

1506 Zhu J-Y, Zhou L-F, Li Y-K, et al (2017) In vivo near-infrared fluorescence imaging of amyloid- $\beta$   
1507 plaques with a dicyanoisophorone-based probe. *Anal Chim Acta* 961:112–118 . doi:  
1508 10.1016/j.aca.2017.01.017

1509 Zimmermann T, Marrison J, Hogg K, O'Toole P (2014) Clearing up the signal: spectral imaging  
1510 and linear unmixing in fluorescence microscopy. *Methods Mol Biol* 1075:129–48 . doi:  
1511 10.1007/978-1-60761-847-8\_5

1512 Zimmermann T, Rietdorf J, Girod A, et al (2002) Spectral imaging and linear un-mixing  
1513 enables improved FRET efficiency with a novel GFP2-YFP FRET pair. *FEBS Lett* 531:245–9

1514 Zinselmeyer BH, Dempster J, Wokosin DL, et al (2009) Chapter 16. Two-photon microscopy  
1515 and multidimensional analysis of cell dynamics. *Methods Enzymol* 461:349–78 . doi:  
1516 10.1016/S0076-6879(09)05416-0

1517 Zipfel WR, Williams RM, Christie R, et al (2003a) Live tissue intrinsic emission microscopy  
1518 using multiphoton-excited native fluorescence and second harmonic generation. *Proc*

- 1519 Natl Acad Sci U S A 100:7075–80 . doi: 10.1073/pnas.0832308100
- 1520 Zipfel WR, Williams RM, Webb WW (2003b) Nonlinear magic: multiphoton microscopy in the  
1521 biosciences. Nat Biotechnol 21:1369–77 . doi: 10.1038/nbt899
- 1522 Zong W, Wu R, Li M, et al (2017) Fast high-resolution miniature two-photon microscopy for  
1523 brain imaging in freely behaving mice. Nat Methods. doi: 10.1038/nmeth.4305
- 1524