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

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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- β 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^{T2}) 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⁻, Ca²⁺) (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- β 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- β 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 (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 Ca^{2+} through voltage-gated channels, as
313 well as the release of Ca^{2+} from intracellular stores (Kandel et al. 2000), and fluctuations in
314 free 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 Ca^{2+}
316 are a robust indicator of action potential firing in neurons, fluorescent Ca^{2+} indicators have
317 become powerful tools for recording neural activity with excellent spatial and temporal
318 resolution. Additionally, different types of Ca^{2+} signaling events have been studied in
319 astrocytes (Srinivasan et al. 2015) and in cardiomyocytes (Herron et al. 2012).

320 In general, Ca^{2+} imaging relies on a fluorescent sensor compound that is introduced into
321 neurons (or other cells) and that, when bound to Ca^{2+} , changes its fluorescent properties. The
322 two primary classes of 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 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 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 Na^+ . In contrast to Ca^{2+}
366 indicators, Na^+ indicators are designed to measure 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 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 Na^+ in neurons (Senatorov et al. 2000). More recently, a greater
372 sensitivity for fast 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 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.

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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 (σ_2) and quantum yield (ϕ), 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 μm 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 ¹: 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 ²: 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.

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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**.

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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.

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692

693 **Tables**

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

Technique	Target	Probe
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- β plaques	<i>e.g. SAD1 (Heo et al. 2013)</i>
Intravenous administration	Astrocytes	Sulforhodamine 101 (Nimmerjahn et al. 2004), sulforhodamine B (Vérant et al. 2013)
	Blood vessels	<i>e.g. Rhodamine B dextran, quantum-dots (Ricard et al. 2016a)</i>
	Arteries	Alexa Fluor 633 (Shen et al. 2012)
	Amyloid- β 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 ²⁺ indicators (GECIs)
	Membrane potential (V _m)	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</i> (Garaschuk et al. 2006))
	Membrane potential (V _m)	Voltage-sensitive dyes (<i>e.g. ANNINE-6</i> (Kuhn et al. 2008), RH-1692 (Murphy et al. 2008))

696
 697

698 **Table 2** Biophysical properties of two-photon–suitable non-specific probes : peak wavelength
699 of two-photon action cross-section (λ_{2PA}) ; peak two-photon action cross-section ($\sigma_2\phi$) ; peak
700 wavelength of molecular brightness ($\lambda_{\epsilon_{max}}$) ; peak molecular brightness (ϵ_{max}) ; fluorescence
701 wavelength (λ_{fluo}). Probes discussed in the text are in bold.
702 (1) : calculated from the σ_2 and ϕ values
703 (2) : data from commercial provider
704 * : data from http://www.drbio.cornell.edu/cross_sections.html (accessed 10/24/2017)

Probe	λ_{2PA} [nm]	$\sigma_2\phi$ [GM]	$\lambda_{\epsilon_{max}}$ [nm]	ϵ_{max} [kcpm]	λ_{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)
Alexa Fluor 633			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)
Cascade Blue	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)
CellTracker Red	~1080				602 (2)		Figure 2D in (Rakhymzhan et al. 2017)
CFP	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)
dsRed	> 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)
eCFP	857	12			476		Figure S1 in (Drobizhev et al. 2011)
eGFP (pH 8)	927	30			510		Figure S1 in (Drobizhev et al. 2011)
eqFP650	1112	8.5			646		Figure S1 in (Drobizhev et al. 2011)
eqFP670	1120	1.3			661		Figure S1 in (Drobizhev et al. 2011)
Evans Blue	850				680 (Saria and Lundberg 1983)		– (Bennewitz et al. 2014)
eYFP	960	25			527 (Merzlyak et al. 2007)		– (Table 2) (Blab et al. 2001)
Fluorescein (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)
GFP	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)
mKate2	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)
mOrange2	~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)
Rhodamine 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)
tdTomato	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)
Venus	~965, ~1015	~19, ~15			528 (Nagai et al. 2002)		Figure 7B in (Hashimoto et al. 2010)
VivoTag 680	820				688		– (Swirski et al. 2007)
YFP	960	228			527		*

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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 (λ_{2PA}) ; peak two-photon
709 action cross-section ($\sigma_2\phi$) ; peak wavelength of molecular brightness ($\lambda_{\epsilon_{max}}$) ; peak molecular
710 brightness (ϵ_{max}) ; fluorescence wavelength (λ_{fluo}). Probes discussed in the text are in bold.
711 (1) : calculated from the σ_2 and ϕ values
712 (2) : data from commercial provider

Probe	λ_{2PA} [nm]	$\sigma_2\phi$ [GM]	$\lambda_{\epsilon_{max}}$ [nm]	ϵ_{max} [kcp/sm]	λ_{fluo} [nm]	Comment	Reference
Alexa Fluor 633			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)
Sulforhodamine 101	910	118	900	38.4	~605 (2)	Astroglia (Nimmerjahn et al. 2004)	Figure 5 in (Mütze et al. 2012)
Sulforhodamine B	810				~586 (2)	Astroglia	Figure 2A in (Appaix et al. 2012)

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715 **Table 4** Biophysical properties of two-photon–suitable environment sensing probes: peak
716 wavelength of two-photon action cross-section (λ_{2PA}) ; peak two-photon action cross-section
717 ($\sigma_2\phi$) ; fluorescence wavelength (λ_{fluo}). Probes discussed in the text are in bold.
718 (1) : calculated from the σ_2 and ϕ values
719 (2) : data from commercial provider

Probe	λ_{2PA} [nm]	$\sigma_2\phi$ [GM]	λ_{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)

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722 **Table 5** Biophysical properties of two-photon-suitable probes in the diseased brain: peak
723 wavelength of two-photon action cross-section (λ_{2PA}) ; peak two-photon action cross-section
724 ($\sigma_2\phi$) ; fluorescence wavelength (λ_{fluo}). Probes discussed in the text are in bold.
725 (1) : calculated from the σ_2 and ϕ values
726 (2) : data from commercial provider

Probe	λ_{2PA} [nm]	$\sigma_2\phi$ [GM]	λ_{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- β aggregates)	900	118	675 / 635	Amyloid- β plaques, penetrates BBB	Figure S7 in (Zhu et al. 2017)
Gatifloxacin	700		~510	Fluoroquinolone antibiotics	Figure 1A in (Lee et al. 2016)
MeO-X04 (in PBS / EtOH)	720	10 / 75	452 / 444	Amyloid- β plaques (Klunk et al. 2002)	– (Table S1) (Heo et al. 2013)
MNAH	820		536	Imaging of mitochondrial singlet oxygen	– (Liu et al. 2016)
Moxifloxacin	700		~530	Fluoroquinolone antibiotics	Figure 1A in (Lee et al. 2016)
PIB (in PBS / EtOH)	740	45 / 40	431 / 417	Amyloid- β plaques	– (Table S1) (Heo et al. 2013)
SAD1 (in PBS / EtOH)	750	10 / 170	497 / 465	Amyloid- β plaques	– (Table S1) (Heo et al. 2013)

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729 **Table 6** Biophysical properties of two-photon–suitable functional probes : peak wavelength
730 of two-photon action cross-section (λ_{2PA}) ; peak two-photon action cross-section ($\sigma_2\phi$) ; peak
731 wavelength of molecular brightness ($\lambda_{\epsilon_{max}}$) ; peak molecular brightness (ϵ_{max}) ; fluorescence
732 wavelength (λ_{fluo}). Probes discussed in the text are in bold.
733 (1) : calculated from the σ_2 and ϕ values
734 (2) : data from commercial provider
735 * : data from 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	λ_{2PA} [nm]	$\sigma_2\phi$ [GM]	$\lambda_{\epsilon_{max}}$ [nm]	ϵ_{max} [kcpsm]	λ_{fluo} [nm]	Comment	Reference
Calcium indicators							
ACa1 (-Ca ²⁺ / +Ca ²⁺)	– / 780	– / 110			498 / 498		Figure 2A in (Kim et al. 2008a)
ACa2 (-Ca ²⁺ / +Ca ²⁺)	– / 780	– / 90			495 / 495		Figure 2A in (Kim et al. 2008a)
ACa3 (-Ca ²⁺ / +Ca ²⁺)	– / 780	– / 95			500 / 517		Figure 2A in (Kim et al. 2008a)
ACaL (-Ca ²⁺ / +Ca ²⁺)	– / 780	– / 90			500 / 502		Figure 1B in (Mohan et al. 2009)
ACaLN (-Ca ²⁺ / +Ca ²⁺)	– / 750	– / 20			494 / 497		– (Table 1) (Lim et al. 2011a)
BCaM (-Ca ²⁺ / +Ca ²⁺)	– / 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 ²⁺)	870	95			615 (2)		*
Calcium Green (+Ca ²⁺)	820, 960	46, 57			531 (2)		*
Calcium Orange (+Ca ²⁺)	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)		*
Fluo-4	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)
Fura-2 +Ca ²⁺)	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 ²⁺ / +Ca ²⁺)	980	0.126 / 0.33 *			515 (Chen et al. 2013)		Figure 4I in (Helassa et al. 2015)
GCaMP3bright (-Ca ²⁺ / +Ca ²⁺)	950	0.02 / 0.7 *			~515	high fluorescence dynamic range	Figure 4I in (Helassa et al. 2015)
GCaMP3fast (-Ca ²⁺ / +Ca ²⁺)	990	0.014 / 0.2 *			~515	fast Ca ²⁺ 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)
Indo-1	730				490 / 405 (Cahalan et al. 2002)		* and Figure 3A in (Wang and Yeh 2012)
jRCaMP1a	~1070	~8			~593	OPO-friendly	Figure 2S1B in (Dana et al. 2016)
jRCaMP1b	~1080	~11			~593	OPO-friendly	Figure 2S1B in (Dana et al. 2016)
jRGECO1a	~1070	~7			~593	OPO-friendly	Figure 2S1B in (Dana et al. 2016)
K-GECO1 (-Ca ²⁺ / +Ca ²⁺)	~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)
Oregon Green BAPTA-1	800	24			523		Figure 2A in (Kim et al. 2008a)
RCaMP1a (-Ca ²⁺ / +Ca ²⁺)	1070 / 1070			– / 5.9	594 / 595		– (Table 3) (Akerboom et al. 2013)
RCaMP1c (-Ca ²⁺ / +Ca ²⁺)	1070 / 1070			– / 7.3	597 / 595		– (Table 3) (Akerboom et al. 2013)
RCaMP1d (-Ca ²⁺ / +Ca ²⁺)	1070 / 1070			– / 7.7	597.5 / 592.5		– (Table 3) (Akerboom et al. 2013)
RCaMP1f (-Ca ²⁺ / +Ca ²⁺)	1070 / 1070			– / 8.2	597 / 591.5		Figure 2F in (Akerboom et al. 2013)
RCaMP1h	~1070	~27			595		#
R-GECO1 (-Ca ²⁺ / +Ca ²⁺)	1065 / 1065			– / 3.8	598 / 588		– (Table 3) (Akerboom et al. 2013)
Other ions							
6-CO2H-ZAP4 (-Zn ²⁺ / +Zn ²⁺)	<700, >1000	– / 86			527 / 523	Zinc	Figure 2B in (Khan et al. 2014)
ANa1 (+Na ⁺)	780	95			500	Sodium	Figure 1C in (Kim et al.

							2010b)
Asante NaTRIUM Green-2 (ANG-2) (-Na⁺ / +Na⁺)	780	0.84 / 5.7			542	Sodium	Figure 2 in (Roder and Hille 2014)
AZn1 (-Zn ²⁺ / +Zn ²⁺)	- / 780	- / 95			496 / 498	Zinc	Figure 1B in (Kim et al. 2008b)
AZn2 (-Zn ²⁺ / +Zn ²⁺)	- / 780	- / 110			494 / 499	Zinc	Figure 1B in (Kim et al. 2008b)
AZnE1 (-Zn ²⁺ / +Zn ²⁺)	- / 780	- / 86			502 / 503	Zinc	Figure 2A in (Danish et al. 2011)
AZnE2 (-Zn ²⁺ / +Zn ²⁺)	- / 780	- / 86			503 / 504	Zinc	Figure 2A in (Danish et al. 2011)
AZnM1 (-Zn ²⁺ / +Zn ²⁺)	- / 780	- / 88			501 / 504	Zinc	Figure 2A in (Danish et al. 2011)
AZnM2 (-Zn ²⁺ / +Zn ²⁺)	- / 780	- / 86			504 / 504	Zinc	Figure 2A in (Danish et al. 2011)
AZnN (-Zn ²⁺ / +Zn ²⁺)	- / 780	- / 89			500 / 502	Zinc	Figure 2A in (Danish et al. 2011)
FMg1 (-Mg ²⁺ / +Mg ²⁺)	- / 740	- / 87			540 / 540	Magnesium	Figure 1C in (Dong et al. 2012)
FMg2 (-Mg ²⁺ / +Mg ²⁺)	- / 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 ²⁺ / +Zn ²⁺)	700 / 700	304 / 565 (1)			465 / 550	Zinc	Figure 1A in (Li et al. 2017)
SBFI (+Na ⁺)	780	20			539	Sodium	Figure 1C in (Kim et al. 2010b)
Sodium Green(+Na ⁺)	800	30			532	Sodium	Figure 1C in (Kim et al. 2010b)
SZn-Mito (-Zn ²⁺ / +Zn ²⁺)	- / 760	- / 75			500 / 493	Zinc	Figure 1B in (Masanta et al. 2011)
SZn2-Mito (-	- /	- /			536 / 536	Zinc	Figure 1B in

Zn ²⁺ / +Zn ²⁺)	750	155					(Baek et al. 2012)
SZnC (-Zn ²⁺ / +Zn ²⁺)	750 / 750	16 / 92			499 / 499	Zinc (Golgi-localized)	Figure 2D in (Singh et al. 2015)
— Schwarze 2015 Probe 1 (DMSO / K ⁺ / Na ⁺)	860	3.1 / 9.4 / 3.4			511	Potassium / Sodium	Figure 2B in (Schwarze et al. 2015)
— Schwarze 2015 Probe 2 (DMSO / K ⁺)	840	5.7 / 16.1			493	Potassium	– (Table 1) (Schwarze et al. 2015)
Voltage-sensitive dyes							
ANNINE-6	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)
di-3-ANEPDPHQ	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)
RH-1692	~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)

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

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

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746 **Table 8** Biophysical properties of two-photon–suitable probes specific of sub-cellular
747 structures : peak wavelength of two-photon action cross-section (λ_{2PA}) ; peak two-photon
748 action cross-section ($\sigma_2\phi$) ; peak wavelength of molecular brightness ($\lambda_{\epsilon_{max}}$) ; peak molecular
749 brightness (ϵ_{max}) ; fluorescence wavelength (λ_{fluo}). Probes discussed in the text are in bold.
750 (1) : calculated from the σ_2 and ϕ values
751 (2) : data from commercial provider
752 * : data from http://www.drbio.cornell.edu/cross_sections.html (accessed 10/24/2017)

Probe	λ_{2PA} [nm]	$\sigma_2\phi$ [GM]	λ_{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 ₃ CN)	750	258	593	Lysosomes	Figure S2 in (Zhang et al. 2018)
ASiR2 (in CH ₃ CN)	760	237	601	Lysosomes	Figure S2 in (Zhang et al. 2018)
ASiR3 (in CH ₃ CN)	760	160	606	Lysosomes	Figure S2 in (Zhang et al. 2018)
ASiR4 (in CH ₃ CN)	760	179	608	Lysosomes	Figure S2 in (Zhang et al. 2018)
ASiR5 (in CH ₃ CN)	750	189	609	Lysosomes	Figure S2 in (Zhang et al. 2018)
ASiR6 (in CH ₃ CN)	750	193	601	Lysosomes	Figure S2 in (Zhang et al. 2018)
ASS (H ₂ 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 ₂ 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)
Quinacrine	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 (λ_{2PA}) ; peak two-photon action cross-section
756 ($\sigma_2\phi$) ; peak wavelength of molecular brightness ($\lambda_{\epsilon_{max}}$) ; peak molecular brightness (ϵ_{max}) ;
757 fluorescence wavelength (λ_{fluo}).

758 (1) : calculated from the σ_2 and ϕ values

759 (2) : data from commercial provider

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