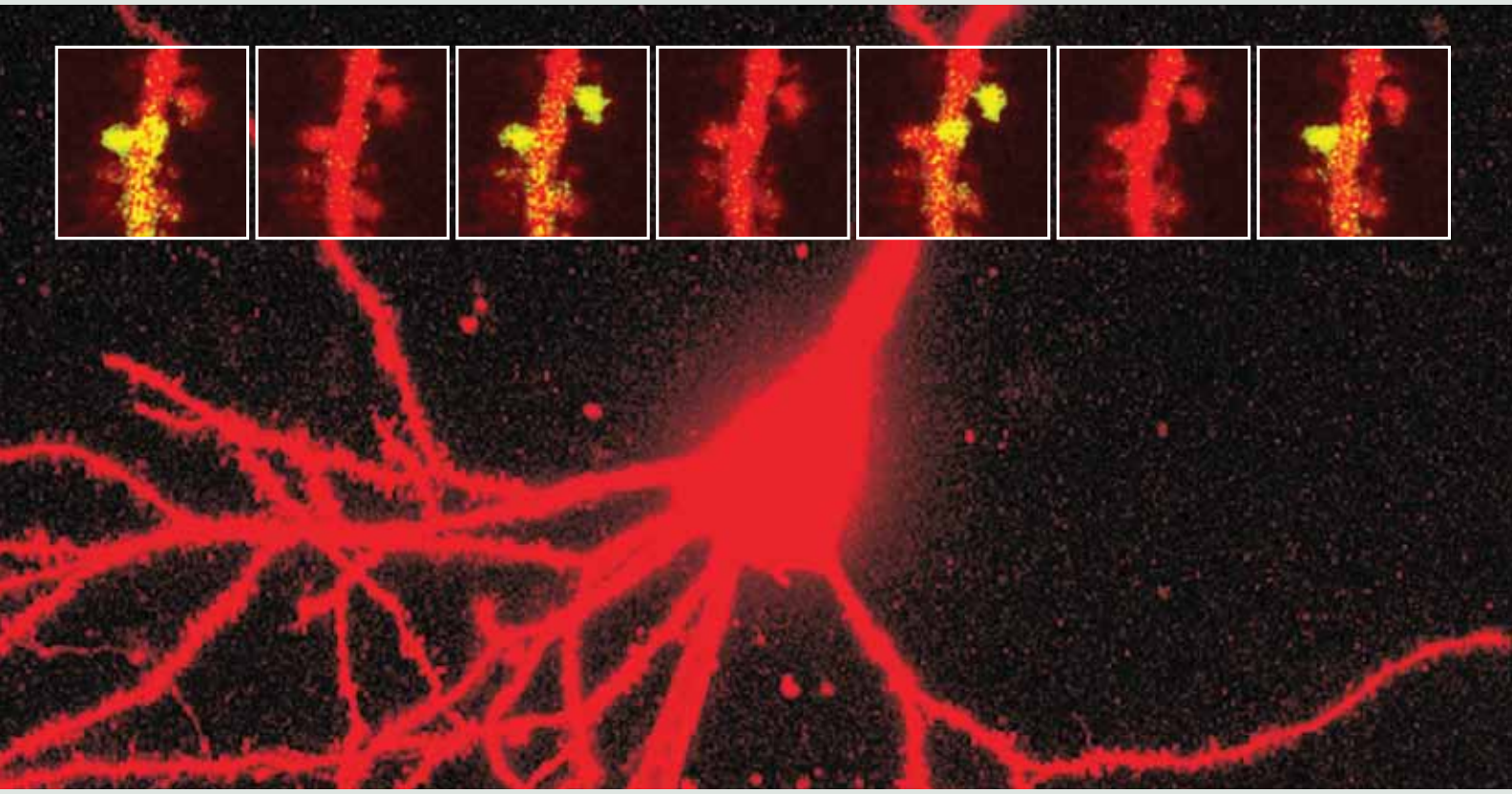

Neurons in focus

TWO-PHOTON EXCITATION IMAGING IN LIVE NEURONAL TISSUE.

Nowhere is preserving native cellular context more important than in studies of the nervous system. This intricate 3D structure is at the core of all of the complex functions we observe—disrupt it, and function is altered. Despite the easy access provided by dissociated neurons in culture, especially in optically based investigations such as fluorescence-based calcium imaging [1], the need for more contextual and system-level analysis has driven the development of techniques capable of exploring tissue slices and even the whole brain. Two-photon excitation (TPE) of fluorescent sensors is one approach giving researchers access to deeper layers of functioning cortex (Figure 1). This article reviews data and observations from the literature about the dye choices compatible with TPE, describes what to consider in making those choices, and lists protocols to optimize their use.

Figure 1 (above). Monitoring synaptically evoked calcium transients by two-photon laser-scanning microscopy in intact brain tissue using fluorescent dyes. A CA1 rat pyramidal neuron was microinjected with 300 µM fluo-5F (Cat. No. F14222), a calcium indicator (K_d 800 nM), and 20 µM Alexa Fluor® 594 hydrazide (Cat. No. A10442). Both dyes were excited at 800 nm using two-photon excitation. In the film insets, several frames of calcium transients are shown following individual evoked action potentials in presynaptic axons (evident by the appearance of a yellow-fluorescent signal against the Alexa Fluor® 594 dye–labeled red-fluorescent background). Using a ratio of the two signals, absolute calcium concentration in individual dendritic spines can be calculated. Such high-resolution optical imaging combined with electrophysiology allows hundreds of transmission events to be recorded at identified synapses, providing temporal and spatial precision that would have been unimaginable just a decade ago. Image courtesy of Thomas Oertner, FMI Institute for Biomedical Research, Basel, Switzerland, and Karel Svoboda, HHMI, Janelia Farms Research Institute, Virginia, US.

Advantages of TPE for live-tissue analysis

The practical application of TPE microscopy has advanced considerably through the pioneering work of Watt Webb and his colleagues during the last two decades [2,3]. The principle of TPE was first predicted by Maria Göppert-Mayer in 1931 [4], and it is after her that the TPE unit of absorption cross-section or excitation efficiency is named. It took another three decades and the invention of the pulsed ruby laser, followed by the invention of TPE laser scanning microscopy in 1990 [2], to see TPE emerge as a practical application. The principal advantages are increased depth of resolution (due to the use of near infrared light—700 to 1,100 nm), narrow focal plane of the excitation volume (less than 100 µm), and reduced photobleaching of dye above and below the imaged region (~0.1 μm^3) [5]. Thus high-resolution, 3D images of depths up to 1 mm (up to 2 mm in some tissues) can be achieved, compared to less than 200 µm for visible light–based approaches [6] (Figure 2).

The science behind TPE is well reviewed elsewhere [7], but can be conceptualized as a summing of the excitation energies through two long-wave photons (e.g., 1,000 nm) that are focused and concentrated at discrete, small focal volumes (Figure 3). Concentrated in time and space, the two weak photons can sum their energies and excite fluorophores that normally have excitation maxima half that value. Since the excitation beam is concentrated in one focal plane, often as small as 0.1 μm^3, there is reduced photobleaching above and below the plane of interest, which eliminates phototoxicity. Thus TPE is rapidly becoming a preferred excitation source for physiological analysis of live tissue.

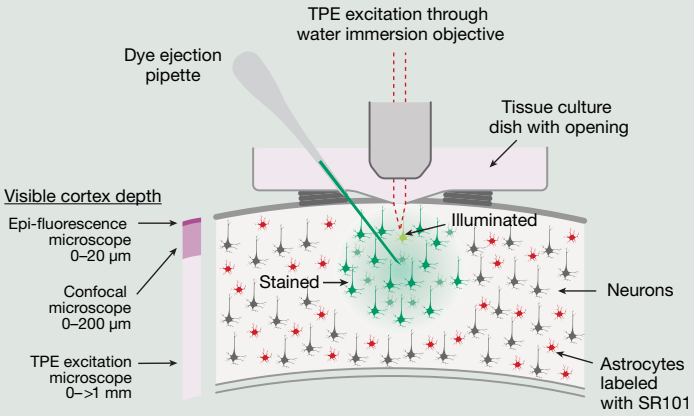

Figure 2. Schematic diagram showing a two-photon excitation (TPE) imaging setup and the depths of resolution of commonly used imaging techniques.

Choosing a fluorophore

In general, any bright one-photon dye can be used in TPE imaging if a somewhat blue-shifted excitation maximum is used (for a complete survey, see the excellent reviews in references 7 and 8). Table 1 lists specific dyes that have been shown to be useful in TPE experiments.

Qdot® nanocrystals have remarkable TPE cross-sectional areas estimated to be three orders of magnitude greater than the best fluorescent dyes or fluorescent proteins. Depending on the size of the Qdot® nanocrystal used, various fluorescent colors can be →

Figure 3. An experiment illustrating ordinary (single-photon) excitation of fluorescence and two-photon excitation. The cuvette contains a solution of the dye safranin O, which normally emits yellow light when excited by green light. The upper lens focuses green (543 nm) light from a CW helium–neon laser into the cuvette, producing the expected conical pattern of excitation (fading to the left). The lower lens focuses pulsed infrared (1,046 nm) light from a neodymium–YLF laser. In two-photon absorption, the excitation is proportional to the square of the intensity; thus, the emission is confined to a small point focus (see arrow), which can be positioned anywhere in the cuvette by moving the illuminating beam. Image contributed by Brad Amos, Science Photo Library, London.

generated from a single wavelength of excitation, allowing multiplexing experiments to be performed relatively easily and without concern for phototoxicity or photobleaching. The many variations of fluorescent proteins, which also possess excellent cross-sectional absorption areas, are also readily adaptable to TPE.

Multi-cell bolus labeling (MCBL)

The simplest way to label cells is to use a micropipet to place dye in the region of interest in a technique called multi-cell bolus labeling (MCBL, Figure 2). Easier than achieving either whole-cell patch clamp or intracellular contact, MCBL can be used to deliver calcium-sensing dyes and dyes that selectively label astrocytes (sulforhodamine 101 (SR101) or its fixable version, Texas Red® hydrazide) in one 5-second injection pulse [9,10]. Many cells stained in this way are observed to have taken up the dye within 20 minutes [11].

This approach offers two main benefits: (1) individual cells (micro) as well as groups of cells (macro) can be studied in the same field; and (2) signal can be precisely attributed to astrocytes or neurons. This approach is effective in lower vertebrates and mammals, including whole alert mice and rats, at dye concentrations as low as 100 µM [8]. For this application, the dyes chosen must be cell permeant. Oregon Green® 488 BAPTA-1 AM has been used, but fluo-4 and others can be considered, and, when used with Pluronic® F-127 (Invitrogen Cat. No. P6867) (BASF Corporation), their uptake is increased. It is necessary to consider various outcomes when optimizing the experimental setup: for example, signal gain vs. signal-to-noise ratio, speed of response, the binding affinity of the dye, and color choice. These attributes are summarized in Tables 1 and 2, and a range of options exists. For example, Oregon Green® 488 BAPTA, when equimolar to fluo-4, is three times brighter, so three times less excitation could be used to achieve the same response. Reduced light excitation is a key factor in maintaining cell health. Oregon Green® 488 BAPTA also produces faint staining at low calcium or pre-stimulation levels, which can be helpful in finding the cells. But fluo-4 and its variants are used when a much bigger signal change is needed. For example, 100 µM of fluo-4 or fluo-5F can provide as much as a 50% signal increase in pyramidal cells [7]. For on/off sensing at individual neurons, Oregon Green® 488 BAPTA is a good choice, but for submicron regions like dendritic spines, the greater signal change with fluo-4 is preferred.

Intracellular injection

While more difficult to perform, whole-cell patch clamping and delivery of dyes into cells via microelectrodes allow more precise calcium ion concentration determinations, control of electrical behavior of the

Table 1. Excitable fluorophores for calcium detection for use in two-photon excitation (TPE) microscopy.

	Fluorophore	K_d in solution at 22–24°C*	Φ TPE cross-section†	TPE optimal excitation	Emission
Blue and green indicators	Oregon Green® 488 BAPTA-1 and -2	170 nM	ND	810–880 nm	530 nm
	Fluo-3	325 nM	10 GM/5 GM	810 nm	526 nm
	Fluo-4	345 nM	ND	800 nm	516 nm
	Fluo-5F	2.3 µM	ND	810 nm	520–530 nm
	Fluo-4FF	9.7 µM	ND	810 nm	516 nm
	Calcium Green™-1 +/– Ca	190 nM	30 GM/2 GM	820 nm	530 nm
	Fura-2 +/– Ca	140 nM	6 GM/0.2 GM	800 nm	505/362 nm
	Indo-1 +/– Ca	230 nM	3.5 GM/1.5 GM	590/700 nm	490/400 nm
Red indicators	X-rhod-1	0.7 µM	ND	900 nm	602 nm
	X-rhod-5F	1.6 µM	ND	770–800 nm	603 nm
	X-rhod-FF	17 µM	ND	ND	603 nm
	Rhod-FF	19 µM	ND	ND	603 nm

* K_d can change inside cells (see reference 11). Data shown are according to Molecular Probes data taken at 22°C. † Expressed in Göppert-Mayer Units (GM); 1 GM = 10^{-50} cm^4 s. For a more complete listing of TPE experiment–compatible dyes, see **www.invitrogen.com/bp60**. **ND** = Not determined.

Table 2. Other useful stains for use in two-photon excitation (TPE) microscopy.

Stain	Target	Φ TPE cross-section*	TPE optimal excitation	Emission
CoroNa™ Green	NA	ND	770 nm	516 nm
CoroNa™ Red	NA	ND	~1,000 nm	570 nm
Sulforhodamine 101 (SR101) or Texas Red® hydrazide (fixable version of SR101)	Astrocytes	ND	840–890 nm	605 nm
Alexa Fluor® 594 hydrazide	Ca^{2+}-insensitive intracellular marker	ND	810 nm	594 nm
Fluorescent proteins	General expression tag	100–200 GM	800–1,030 nm	505–610 nm
Rhodamine B	General cell stain	100–200 GM	840 nm	600 nm
Lucifer yellow CH L453, L682, L1177	Gap junctions	ND	850 nm	533 nm
DAPI	DNA/nuclei	0.16 GM	700 nm	450 nm
FM® 1-43	Vesicle recycling	ND	840 nm	575–600 nm†
Qdot® nanocrystals	NA	Up to 47,000 GM	Broad	8 colors from 545–800 nm

* Expressed in Göppert-Mayer Units (GM); 1 GM = 10^{-50} cm^4 s. † Strongly environment dependent. **NA** = Not applicable. **ND** = Not determined.
For a more complete listing of TPE experiment–compatible dyes, see **www.invitrogen.com/bp60**.

cell, and delivery of intracellular agonists to second messengers. High affinity dyes such as fluo-4, fluo-5F, X-rhod-1, or X-rhod-5F are used to detect single action potentials or small synaptically evoked signals whereas lower affinity dyes such as fluo-4FF, X-rhod-FF, or rhod-FF are used to sense trains of action potentials or larger synaptically evoked signals [12]. It has been noted that the apparent affinities of nearly all dyes will be affected by other ions already free in the sample; thus the affinities offered in the table generally need to be halved to estimate their behavior in cells. Moreover, the interaction of calcium with the dye will also impact the free calcium levels, competing for ions that might otherwise be signaling.

While ratiometric dyes (e.g., the excitation ratio fura dyes or the emission ratio indo-1 dyes) are optimal for deriving accurate calcium concentrations, TPE does not readily lend itself to this approach. A far easier approach is to co-inject a red dye, such as Alexa Fluor® 594 dye, and monitor ratio changes in levels of indicator dye against the constant value of the Alexa Fluor® 594 dye for that cell [7]. One group has shown that multiple sensing dyes can be co-injected, which opens a diverse range of indicator options. In their experiments, they filled neurons via intracellular injection with a sodium-sensing reagent, CoroNa™ dye, and a red-shifted calcium indicator, X-rhod-5F. Both can be excited by 770 nm light, conveniently far from the wavelengths used to excite Green Fluorescent Protein (GFP)-labeled neurons (commonly employed to observe cell morphology and to identify candidate cells), yet their emissions are monitored separately. In doing so, they found that currents for sodium and calcium were co-distributed at action potential initiation sites in cochlear interneurons [13].

The future of TPE imaging

Using the TPE technique, experiments considered impossible several years ago are now moving ahead. Possibly the most remarkable and complex of these approaches involves analysis of the contribution of NMDA-induced calcium transients to secondary downstream signaling such as RAS [14] at the level of individual dendritic spines of hippocampal neurons in brain slices. NMDA pulses are delivered with regional accuracy by using the same TPE to uncage MNI-caged glutamate [15]. Alternatively, fluorescent protein–tagged protein pairs can be co-injected and FRET interactions studied through lifetime changes, as regulatory elements combine in response to ion fluxes [13]. Tools like these, available from Invitrogen (Tables 1 and 2), are allowing researchers to explore the molecular substrates of learning and memory at the most basic structural level—the minute spines that decorate dendrites throughout the brain. Learn more at **www.invitrogen.com/bp60**. ■

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