Targeted Optical Probing of Neuronal Circuit Dynamics Using Fluorescent Protein Sensors

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Key Words

Green fluorescent protein • Optical indicator • Biosensor •
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Abstract

Interest in non-invasive methods for optical probing of neuronal electrical activity has been ongoing for several decades and methods for imaging the activity of single or multiple individual neurons in networks composed of thousands of neurons have been developed. Most widely used are techniques that use organic chemistry-based dyes as indicators of calcium and membrane potential. More recently a new generation of probes, genetically encoded fluorescent protein sensors, have emerged for use by physiologists studying the operation of neuronal circuits. In this review we describe the advance of these emerging optical techniques and compare them with more conventional approaches.

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Introduction

Optical imaging methods for monitoring physiological activities are powerful tools for a variety of applications. They are of particular importance in the study of the nervous system where different compartments of in-

dividual neurons, local circuits of many neurons and distinct brain areas, are simultaneously active. Optical probing of neuronal circuit dynamics aims at understanding the interactions between large populations of neurons that participate in specific circuits by monitoring the activity of thousands of individual neurons simultaneously. Optical methods using organic chemistry-based dyes, such as voltage-sensitive dyes and calcium-sensitive dyes, have been widely applied in various fields of neuroscience for more than two decades. With the more recent development of fluorescent protein-based probes, targeting of specific cell populations has become feasible. Specific labeling of genetically defined cell types facilitates a variety of new optical approaches, such as whole-field fluorescence imaging of the dynamics of specific neuron populations. In this review we describe the advance of these emerging optical techniques on the background of more conventional approaches.

Optical Probing of Neuronal Circuit Dynamics

Classical system electrophysiology, like the work of Hubel and Wiesel, was based on the use of microelectrodes to record extracellularly, and more recently intracellularly, the electrical signals generated by individual neurons in the living animal. While it is clear that the operation of neuronal circuits is based on the transmis-

Fig. 1. The various levels of neuronal organization are associated with a spatial scale and function is associated with a temporal scale. Available experimental techniques cover areas in a plot of these two dimensions. Microelectrode techniques (e.g. patch clamp techniques) cover the cellular spatial scale and the time scale from millisecond – or below – to an hour or more. FMRI, on the upper right end covers whole brains but its spatial resolution is limited at about 1 mm, while the temporal resolution starts at about 1 s. Optical imaging covers the molecular to the behavioral scale with sub-millisecond to day-long temporal coverage.

sion of these signals to other synaptically connected neurons, microelectrodes are generally incapable of resolving signal propagation in cellular circuits and hence do not allow a direct observation of distributed information processing. Another limitation of microelectrode techniques is that we usually know little about the identity of the recorded cell (excitatory or inhibitory subtype, etc.).

Thus, significant progress in our understanding of neuronal networks requires the ability to monitor the activity of thousands of individually identified neurons simultaneously. It was realized many years ago that functional optical imaging can provide the required spatial and temporal resolution and coverage (fig. 1).

Modalities of Functional Optical Imaging

Functional optical imaging can, in principle, be performed in at least four modalities: absorption, scattering, chemoluminescence and fluorescence.

Changes in absorption and scattering without exogenous application of a dye are the basis of imaging techniques that are usually referred to as ‘intrinsic imaging’ [1]. There are also dyes that change their absorption as a function of neuronal activity (e.g. calcium- and voltage-dependent absorption dyes). Their application is limited to transparent preparations that allow the passage of high intensity of light with little scattering. Chemoluminescence-based imaging has been a powerful method at the level of single isolated cell preparations but the limited S/N has made application in intact neuronal circuits difficult or prohibitive. Fluorescence is the modality of choice for in vivo and most in vitro optical imaging approaches because: (i) in fluorescence measurements most of the detected light is emitted by the probe with little background signal; (ii) each dye molecule can generate many photons per time unit, and (iii) fluorescence generally is much more sensitive to the environment of the chromophore (a consequence of the relatively long time a molecule stays in an excited state) and, therefore, fluorescent probes are typically more sensitive than absorption probes.

Intrinsic Fluorescent Probes

Functional optical imaging can be done without the need of added dyes since neuronal tissue naturally contains molecules which exhibit activity-dependent fluorescence. Excitation of brain tissue with UV (~350 nm) light excites fluorescence that broadly peaks around 460 nm. The main source of this ‘blue’ tissue ‘autofluorescence’ is nicotinamide adenine dinucleotide (NADH). Neuronal activity promotes oxidative phosphorylation during which NADH is oxidized to the non-fluorescent molecule (NAD⁺). Changes in NADH fluorescence were probably the first optical real-time measure of metabolic activity [2] and many years ago, the usefulness of NAD(P)H fluorescence measurements for monitoring intense and coordinated central nervous system activation had already been demonstrated during seizures [3, 4].

More recently, ‘green’ brain tissue ‘autofluorescence’ has been characterized for monitoring of neuronal activation. The ‘activity-dependent’ fluorescent molecule is most likely flavin adenine dinucleotide [5]. Green autofluorescence optical imaging has been used for mapping activity in the central nervous system in vivo and for investigating the coupling among neurons and glia [6–8].

Fig. 2. Green autofluorescence signals in the cerebellum in vivo. **a** Fluorescence image of cerebellar vermis VI in a living ICR mouse with stimulation electrode (S) placed in the molecular layer. Yellow rectangle indicates cropped region used for color-coded maps of autofluorescence (shown in **b**) in response to a train of stimulation pulses (10 pulses at 100 Hz, 100 μ A). **b, c** Color-coded map of peak autofluorescence signal (**b**) and time-course of $\Delta F/F$ values (**c**) obtained from responsive area (outline shown in **a**) before (ACSF), during (NBQX + D-APV + LY) and after (recovery) superfusion of the mixture of 40 μ M NBQX + 250 μ M D-APV + 200 μ M LY367385. Note that the green autofluorescence signal is significantly decreased when blocking AMPA, NMDA and mGlu1 receptors. **d** Time course of $\Delta F/F$ values obtained with 1, 2, 4, 8 and 10 stimulation pulses (100 Hz, 100 μ A).

In the cerebellum, green autofluorescence optical imaging has been used to investigate the flow of excitation in the mossy fiber-granule cell to Purkinje cell pathway [8]. Figure 2 illustrates an experiment where stimulation of parallel fibers in the cerebellar vermis VI evokes a transverse, ‘beam-like’ response. The green autofluorescence signal was obtained with excitation light at 470–490 nm and emission filtered at 520–540 nm. The signal amplitude increased with the number of stimulation pulses (fig. 2a, d) and was largely depressed after blocking synaptic transmission by adding blockers of glutamate receptors (fig. 2b, c) demonstrating that it is primarily of postsynaptic origin. The ‘beam-like’ response that extends over the entire field of view (~ 1.5 mm) corresponds to the recordings obtained with microelectrodes where beams of activated parallel fibers drove synaptic excitation of their postsynaptic targets, Purkinje cells and molecular layer interneurons [8–10].

It has been noted in several brain regions that the green autofluorescence signals are larger and faster than the blue NADH signals [6, 11]. However, both blue and green autofluorescence signal levels are too low to achieve cellular resolution and the kinetics of both are too slow to monitor fast circuit dynamics.

Organic Fluorescent Probes

Organic chemistry-based calcium- and voltage-sensitive dyes with excellent sensitivity are available for imaging of neuronal circuit dynamics. Organic voltage-sensitive dyes along with ultrafast and low-noise imaging techniques made it, for instance, possible to directly visualize the shape and propagation of action potentials in axons of mouse neurons [12]. In the experiment illustrated in figure 3a and b, cerebellar slices were cut coplanar with the surface of cerebellar lobulars V/VI (fig. 3a) and stained with the voltage-sensitive dye di-4-ANEPPS [13]. A stimulation electrode was placed on the surface of the cerebellum (asterisk in fig. 3a). Delivery of a single electric shock induced a volley of action potentials that propagated in both transverse directions and that was readily monitored by the voltage-sensitive dye.

Organic calcium-sensitive dyes are widely used to image the activities of many neurons, or many sites with a neuron, simultaneously with excellent spatial and good temporal resolution [14, 15]. In the experiment illustrated in figure 3c and d, cerebellar slices were stained with the membrane-permeable calcium indicator Fura-2AM (fig. 3c). This loading procedure successfully stains a large number of cells in a small area in the granule cell layer. Afferent stimulation by an electrode placed into the

Fig. 3. Optical imaging obtained with calcium- and voltage-sensitive dyes. **a, b** Optical imaging of the propagation of action potentials in cerebellar parallel fibers. **a** Fluorescence imaging on lobule V/VI of mouse cerebellar cortex stained with a voltage-sensitive dye. Stimulation electrode was placed at the point indicated by asterisk. **b** Voltage changes optically recorded at times after stimulation as indicated [for details, see 13]. **c, d** Ca^{2+} signals induced by white matter stimulation in Fura-2AM-stained cerebellar slice. **c** Fluorescence image of Fura-2AM-stained cerebellar slice. **d** Time course of $\Delta F/F$ values in response to a 10-pulse stimulus (100 Hz, 100 μA). Trace numbers correspond to cell numbers shown in **c**.

white matter induced calcium responses in various granule cell bodies (fig. 3d).

The most serious limitations of organic fluorescent dyes are the lack of broadly applicable methods that allow for staining large populations of specific cell types within tissue that contains a large number of cell types. As discussed below, genetic techniques to assign probes to specific cell types can overcome these limitations.

Genetically Controlled Cell Staining of Specific Cell Populations

Indicator loading of specific cell populations is possible with sensors whose function is dependent on the presence of a protein. This is because cell type-specific pro-

tein expression can be achieved by the use of appropriate regulatory sequences (promoters). This concept had been proposed many years ago in conjunction with the bioluminescent calcium-sensitive reporter protein aequorin but the light output of bioluminescent probes is rather limited and consumes a co-factor that needs to be applied to the preparation [16]. Genetically encoded fluorescent probes became a powerful approach after the molecular cloning of *Aequorea victoria* green fluorescent protein (GFP) and similar proteins from corals, and their bright spectrally and optically improved variants [17, 18]. A now broadly used approach to generate fluorescent protein-based sensors involves the molecular fusion of a conformation-sensitive GFP-based reporter protein to a second transducer protein that undergoes conformational transitions in response to fluctuations in calcium concentra-

Table 1. Fluorescent sensors evaluated for their ability to monitor action potentials in mammalian brain tissue

	Sensitivity for action potential detection ($\Delta F/F$)	Response time (80% of signal amplitude per action potential)	Components	References
<i>Ca²⁺ sensors</i>				
Non-protein sensors				
Fura-2	>20%/AP burst (upstate)	on: 2 ms, off: >100 ms ^A (34°C)	Stilbene-BAPTA	51–53
Oregon green 488 BAPTA-1	1–10%/1 AP	on: 2 ms, off: >100 ms ^A (34°C)	Fluorescein-BAPTA	14, 53
X-Rhod-5F	20%/1 AP; 100%/20 APs at 20 Hz	on: <1 ms off: >100 ms ^A (24°C)	Rhodamine-BAPTA	54, 53
Genetically encoded sensors				
Yellow Cameleon 3.6	2% (tetanic stimulation)	off: 870 ms	CFP-CaM-M13-YFP	36
G-CaMP2	1%/1 AP 15%/10 AP at 100 Hz	on: ~20 ms off: ~130 ms (35°C)	M13-cpGFP-CaM	
Camgaroo-2	20%/20 AP at 20 Hz		CaM inserted at Y145 of YFP	54
TN-L15	10–20% (spontaneous AP burst)	on: 500 ms, off: 1,330 ms (25°C)	CFP-TnC-YFP	55
TN-XL	3% (44 AP at 20 Hz)	on: 430 ms, off: 240 ms (25°C)	CFP-TnC-cpCitrine	55
<i>Voltage sensors</i>				
Non-protein sensors				
di-4-ANEPPS	0.1–1%/100 mV ^B	on/off: <10 μ s		13
JPW3028	1–6%/100 mV	on/off: <100 μ s	(aminostyryl)pyridinium	56
Hybrid sensors				
hVOS	5–34%/100 mV	on/off: 0.5 ms	GFP/DPA	57
Genetically encoded sensors				
FlaSh ^C	3%	on: ~10 ms, off: ~50 ms	Shaker-GFP	26, 58
SPARC ^C	0.5%	on/off: ~1 ms	skM1-GFP	27
VSFP1	1.8%	on/off: <0.7 ms	Kv2.1(S1–S4)-CFP-YFP	29
VSFP2.1	0.2%	on: ~15 ms, off: ~75 ms	Ci-VSP(VSD)-CFP-YFP	30

AP = Action potential; BAPTA = *bis*-aminophenoxyethane-tetraacetic acid; CaM = calmodulin; CFP = cyan variant of jellyfish green fluorescent protein; cpGFP = circularly permuted GFP; DPA = dipicrylamine; GFP = green variant of jellyfish green fluorescent protein; Off = time constant of sensor response to Ca²⁺ decrease or repolarization; On = time constant of sensor response to Ca²⁺ increase or depolarization; Shaker = *Drosophila* shaker K⁺ channel; Kv2.1(S1–S4) truncated rat Kv2.1 K⁺ channel; skM1 = rat

skeletal muscle Na⁺ channel; TnC = chicken skeletal muscle troponin C; YFP = yellow variant of jellyfish green fluorescent protein; Ci-VSP = *Ciona intestinalis*-voltage-sensor-containing phosphatase.

^A Off time constants depend on indicator concentration.

^B This value is considerably larger in isolated membranes with little background staining.

^C Values extrapolated from non-mammalian preparations.

tion or membrane voltage. The conformation sensitivity of the GFP-based reporter is achieved either via changes in the efficiency of fluorescence resonance energy transfer (FRET) between two spectral variants of fluorescent proteins or via direct effects of single spectrally instable FP variants (e.g. GFP variants with a WT chromophore or circularly permuted variants of GFP). In table 1 we have summarized $\Delta F/F$ values per single or repetitive action potentials (Ca²⁺ probes) or voltage-sensitive (voltage probes), along with response time constants.

Several principal designs for fluorescent protein calcium sensors were explored since the first report of this approach by two independent groups around 10 years ago

[19, 20], including Yellow Cameleon [19], Camgaroo [21], CaMP/Pericam [22, 23], and TN-L15 [24]. Cameleon, Camgaroo and G-CaMP/Pericam use Calmodulin (CaM) and the calmodulin-binding polypeptide M13 (M13) as the calcium sensor, while in TN-L15 troponin C is used for this purpose. Fluorescent reporter proteins of these probes are a pair of cyan (CFP) and yellow (YFP) variants of *A. victoria* fluorescent protein (Yellow Cameleon and TN-L15 [24], a split YFP (Camgaroo) or a circularly permuted fluorescent protein (CaMP/Pericam) [for a detailed overview, see 25].

Three prototypic designs of fluorescent protein voltage sensors have been published to date and termed FlaSh,

Fig. 4. Expression of GCaMP2 in the cerebellar cortex of a pKv3.1/GCaMP2 transgenic mouse. Tangential optical sections of the cerebellar cortex were obtained by two-photon fluorescence imaging (900-nm excitation wavelength) of an in vitro cerebellar macroslice preparation at different depths below the pia surface. **a** Image of the mid-molecular layer with dark structures corresponding to blood vessels (arrows) and interneurons (arrowheads). **b** Image including the Purkinje neuron layer. Purkinje cell bodies appear as dark disks of large diameter (arrowheads). **c** Image of the granule cell layer (180 μm below the pia surface) with densely packed granule cell bodies (arrows) and a few Golgi cell bodies (dark, arrowheads). **d** Image of the granule cell layer at higher resolution. Scale: 25 μm (**a–c**) and 5 μm (**d**).

SPARC and VSFP. Each of them makes use of a membrane protein that can undergo voltage-dependent conformation changes fused to a fluorescent protein receptor. FlaSh was obtained by inserting GFP into the terminus of the *Drosophila* shaker K⁺ channel [26]; SPARC is based on insertion of GFP into a skeletal muscle Na⁺ channel [27]. The application of FlaSh and SPARC is limited by slow kinetics, low sensitivity, and/or a lack of function in mammalian cells [28]. VSFPs were originally designed to exploit the voltage-dependent conformational changes associated with the voltage sensor domain (VSD) of voltage-gated K⁺ channel (Kv) proteins and more recently extended to the use of a VSD of ci-VSP [29, 30]. VSFPs use either FRET [29] or permuted fluorescent proteins [31]. With the exception of VSFP2.1 and its derivatives, most of the currently available fluorescent protein voltage sensors are poorly targeted to the plasma membrane of neurons, so that a major fraction of the fluorescence is sampled from non-responsive GFP [28, 30].

Genetically Modified Mice Expressing FP Sensors

Probing circuit dynamics by means of FP-based sensors requires expression of the sensor protein in intact neuronal tissue. This may be achieved by virus-mediated transfection strategies but genetically modified mice (transgenic or gene targeted) provide the most reproducible experimental preparations. In either case, regulatory sequences (promoters) that drive expression of high levels of protein in specific cell populations are required. The choice of promoters that have been proven of sufficient strength for expression of FP-based probes and desired cell-type specificity is surprisingly limited. A widely used promoter is the Thy1 promoter [32]. However, the pattern of the expressed protein varies considerably between Thy1 mouse lines. In addition to promoters with broad (and varying) expression patterns, regulatory sequences that normally drive proteins that are classically used by neurochemical anatomists and by electrophysiologists to define subtypes of neurons have proven useful. Thus,

Fig. 5. Parallel fiber calcium transients measured in vitro and in vivo using GCaMP2 mice. **a** Fluorescent image of a coronal cerebellar slice from a GCaMP2 transgenic mouse with stimulation electrode (S) placed in the molecular layer. Four stimulation pulses (100 Hz, 100 μ A) induced a longitudinal beam of fluorescence increase. **b** Time course of $\Delta F/F$ values obtained with 1, 2, 4, and 8 stimulation pulses. **c** Fluorescence image of cerebellar vermis VI in a living GCaMP2 mouse with stimulation electrode (S) placed in the molecular layer. Stimulation with four pulses (100 Hz, 100 μ A) induced a beam of fluorescence increase in the cerebellar vermis VI. **d** Time course of $\Delta F/F$ values obtained with 1, 2, 4, and 8 stimulation pulses (100 Hz, 100 μ A).

high levels of neuron-type specific expression of fluorescent proteins have been achieved with promoters for the GABA synthesizing enzyme glutamic acid decarboxylase [33], olfactory receptors [34] and the potassium channel Kv3.1 [35]. More candidate genes have been identified (www.gensat.org/index.html; www.brain-map.org).

Transgenic Mice Expressing the FP Calcium Sensor GCaMP2

The first transgenic mouse strain to express a functional fluorescent protein calcium sensor in well-defined subpopulations of neurons was engineered from the indicator protein GCaMP2 together with regulatory sequences of the Kv3.1 potassium channel [36]. In the cerebellar cortex, for instance, these animals show significant amounts of GCaMP2 expression only in granule cells. Optical sectioning of the cerebellum by two-photon microscopy reveals strong GCaMP2 fluorescence in

granule cell axons in the molecular layer (fig. 4a) and in the cytosolic compartment of granule cell somata in the granule cell layer (fig. 4c, d), whereas no fluorescence is detected from Purkinje neurons and local interneurons (fig. 4a–c) [37]. In the olfactory bulb, GCaMP2 is targeted to mitral cells and subpopulations of periglomerular cells [38].

Physiological experiments using these mice have provided insights as to the unique advantages but also the current limitations of this methodology. GCaMP2 was expressed in cerebellar parallel fibers that are only 0.1–0.3 μ m in thickness, and hence are not accessible to intracellular microelectrode recordings. However, using the transgenic GCaMP2 mouse described above, we demonstrated reliable measurements of calcium transients from beams of cerebellar parallel fibers in response to electrical stimulus in molecular layer both in vitro and in vivo. These GCaMP2 signals are distinct from the green autofluorescence in that they exhibit faster kinetics and are of presynaptic origin. Thus, blockade of postsynaptic

Fig. 6. Principal optical instrumentation for high-resolution functional imaging of neuronal tissue. **a1** Schematic layout of a confocal laser scan microscope (CLSM) showing major optical elements as indicated in the figure. PMT = Photomultiplier tube; NIR = near infrared. **a2** View of the spatial structure of the excitation focus produced by 488-nm excitation light through a diffraction-limited objective with numerical aperture 1.0. The focus is shown in the (z, x)-plane with the focal plane at (z = 0, x). **a3** Effective detection focus in the CLSM obtained by confocal spatial filtering of the excitation focus in **a2**. **b1** Schematic layout of a two-photon laser scan microscope (2pLSM). **b2** Effective two-photon excitation focus in the 2pLSM using 800-nm excitation light. The foci in **a2–b2** were calculated from the effective point spread functions (PSFs) in paraxial approximation. White color is attributed to points where the value exceeds 0.2% of the PSF maximum.

glutamate receptors by application of AMPA, NMDA and mGlu1 receptors antagonist is without effect on the GCaMP2 calcium transients [36, 37]. In the presence of AMPA receptor antagonist, NBQX (40 μM), NMDA receptor antagonist, D-APV (250 μM) and mGlu1 receptor antagonist, LY367385 (200 μM), molecular layer stimulation (4 pulses at 100 Hz, 100 μA) induced a longitudinal beam of fluorescence response representing compound calcium transients in parallel fibers both in coronal slices and the living animal (fig. 5a, c). Compared to the slice experiments performed at room temperature, the in vivo response exhibits a significant decrease in the response decay time constant (fig. 5b, d) [36, 37].

These experiments demonstrated that, because of the precise targeting, calcium imaging from parallel fibers

can be obtained using standard epifluorescence optics and a CCD detector in vitro and in vivo, even though single fibers were not optically resolved. Our experiments with these fluorescent protein calcium sensor mice also highlighted a potential pitfall of green/yellow fluorescent protein sensors, namely interference with green/yellow activity-dependent autofluorescence signals [5, 8, 36]. In the case of the above study, the sensor expression level was sufficiently high so that interference with tissue autofluorescence was negligible. These mice set a benchmark, but encourage further improvement, including sensors with an emission band above 540 nm ('red sensors') for double labeling and to minimize potential interference with intrinsic optical signals.

Optical Instrumentation Considerations

Functional fluorescence imaging requires acquisition of images of the sample using the fluorescence light emitted by a probe. The adequate optical instrumentation depends on the properties of the sample and overall aim of the experiment by no lesser means than, for instance, the appropriate choice of the fluorescence probe itself. One general aspect of all fluorescence imaging methods is the necessity to discriminate efficiently the probe emission from the excitation light. This is generally achieved by spectral filtering employing dielectric filters and/or dispersive optical elements (prisms, gratings, acousto-optical modulators). The appropriate spectral design of these elements is essential for minimizing the crosstalk between the excitation and emission channels, as well as between different emission channels, and hence for the quality of the acquired fluorescence data in any of the imaging modes described below.

The fluorescence image can be obtained by imaging the fluorescent sample under wide-field excitation (e.g. Koehler illumination of the sample with excitation light) onto a camera-type photodetector. Most widely adapted is the epifluorescence configuration where excitation and emission light use the same pathway through the microscope objective. The principal advantages of the wide-field mode are the gain of signal-to-noise (by the multiplex advantage over scanning-type microscopes) and the possibility of obtaining high frame rates (up to 10 kHz using commercial high-speed CCD detectors). Moreover, the wide-field mode is easily integrated into classical digital light microscopes with a few relatively inexpensive additions for fluorescence excitation and spectral filtering. The mode is particularly useful for the detection of cellular and subcellular functional signals from individual neurons in the absence of significant fluorescence background and light scattering within the sample, as well as for the acquisition of very fast voltage signals *in vitro* and *in vivo* [39, 40]. However, because of poor axial resolution, images are blurred by fluorescence from off-focus planes. This effect is overcome in the confocal imaging mode by spatial filtering in the detection path as realized in the confocal laser scan microscope (CLSM, fig. 6a1).

In the CLSM, the probe is excited by a focused laser beam which is scanned across the sample, and the image is reconstructed from the time-correlated signal of fluorescence light passing through a pinhole in the image plane. The combination of point-like excitation and detection (fig. 6a2, a3) enables the CLSM to achieve a superior

spatial, and in particular axial, resolution allowing optical sectioning and thus 3D reconstruction of fluorescent structures in superficial layers of nervous tissue (thickness $<30\ \mu\text{m}$). In deeper layers, e.g. at depths beyond the average ballistic free path of photons in tissue, confocal imaging becomes increasingly inefficient because of the rejection of scattered signal photons at the confocal pinhole. Imaging at higher depth (up to about $1,000\ \mu\text{m}$) is achieved by the two-photon laser scan microscope (2pLSM, fig. 6b1). In the 2pLSM a pulsed near-infrared laser of high peak power is focused into the sample to excite the probe via two-photon absorption [41]. Because of the non-linearity of the 2p absorption process, the probe is excited effectively only within a highly confined 3D volume (see fig. 6b2). Like in the CLSM, images are sequentially generated through scans of the laser focus in the focus plane. In the 2pLSM, however, the image resolution is entirely determined in the excitation path to the great advantage that scattered emission photons can be used for image generation at no loss of 3D resolution. In addition, the near-infrared excitation provides for a longer ballistic penetration length into neuronal tissue as compared to visible light. For these reasons the 2pLSM is commonly utilized for functional fluorescence imaging of live neuronal tissue *in vivo* [42, 43]. More recent developments in microscopy, like confocal endoscopic imaging [44, 45], multifocal and fast 3D scanning [46, 47], fluorescence lifetime imaging [48, 49], and non-diffraction limited microscopy [50], add to the versatility of instrumental tools for specialized applications in analytical neuroimaging.

Conclusion

The development of optical methods to monitor neuronal activities began nearly 35 years ago and enormously progressed in a variety of directions. Further technical advance is required in two main fields: (i) methods for fluorescence imaging at very high frame rates in three dimensions [47], and (ii) further improvements of available probes, in particular genetically encoded voltage sensors, suitable for deep tissue imaging.

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