Applications of two-photon microscopy in the neurosciences

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[Frontiers in Bioscience 10, 2263-2278, September 1, 2005]

APPLICATIONS OF TWO-PHOTON MICROSCOPY IN THE NEUROSCIENCES

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1. ABSTRACT

Thanks to deep tissue penetration of infrared light (IR), two-photon laser scanning microscopy (2PLSM) has become an important tool in modern neuroscience. Examples range from the high-resolution visualization of dendritic spines in single neurons to the monitoring of intracellular Ca^{++} transients in small neural networks. In the present review we give an overview of the current achievements and discuss emerging applications of the technique.

2. INTRODUCTION

Fluorescence microscopy is one of the most powerful tools in cell biology. However, to study the function of neurons in living tissue the poor penetration of light from sources typically used for single-photon excitation is a problem that has to be overcome. A solution is the use of ultra-short pulses of long wavelength beams (near IR) that scatter less light and penetrate deeper into samples. Such light sources have the additional advantage of exciting the fluorophore exclusively in the focal plan, which increases the signal to noise ratio and effectively reduces photobleaching (see also chapter by D.W. Piston). Combined with a scanning device, these new light sources give rise to two-photon laser scanning microscopy (2PLSM), which allows the researcher to study molecular mechanisms in a single neuron as well as in neuronal networks. Although the above listed arguments make a compelling case for the use of 2PLSM in brain tissues, procedures of dye loading are more challenging in such preparations.

In organotypic cultures and *in vivo*, viral (e.g. lentivirus, sindbis virus or semliki forest virus), biolistic gene transfection (gene gun) and electroporation are currently used to induce expression of fluorescent proteins. For example, green fluorescence protein (GFP) constructs can be co-expressed or conjugated to virtually any protein to monitor its localization or movement. With these techniques, the distribution and the expression of the fluorescent protein through the incubation period are stable. However, viral and biolistic transfection efficiencies remain low and these methods are not devoid of toxic or physical damage to the tissue.

An alternative method used in acute slices or *in vivo* is to load fluorescent dyes into a neuron through a patch-clamp pipette. However, this technique limits visualization to a few neurons and relies on the use of soluble dyes. The whole-cell configuration may also interfere with the function studied (e.g. washout of the capability to induce long-term potentiation (LTP)). A solution to this problem is to use sharp electrodes containing a very high concentration of dye in order to maintain the concentration of endogenous cytoplasmic proteins. With some limitations, neurons in thick preparations can also be labelled with cell-permeant dyes. Fluorophores coupled to acetoxymethyl (AM)-esters (1, 2),

(e.g. Fura2-AM, Oregon green BAPTA1-AM) readily enter the cell within minutes where endogenous esterases cleave the AM group rendering the fluorophore membrane impermeant. Non-specificity of AM-loading can be overcome by a localized ejection of the dye close to cellular bodies or axon bundles (3, 4). Ballistic delivery of dextran-conjugated fluorescent indicators to neurons in slices or even *in vivo* is also possible. This method has the advantage of yielding a higher single cell resolution than AM-dye loading (5). Finally, soluble dyes can also cross the membrane with electroporation, but the high voltage applied to allow entry of the charged dye may damage the cell (6, 7). The major inconvenience of diffusion-based loading protocols described here is the equilibration time required for the dye to reach distant compartments of the neuron (typically tens of minutes).

Membrane bound fluorophores such as stiryl dyes have been used to analyse vesicular trafficking or membrane potential variations. For example, FM dyes have recently been used in thick slice preparations to image vesicles that undergo endocytosis and exocytosis. This has become possible due to the development of a chelator that removes non-specific staining (see section 7). Voltage-sensitive dyes are being currently optimized for monitoring network activity (see section 4.1).

For many applications, the best option for imaging *in vivo* and in acute slices is the use of transgenic mice that co-express GFP with the gene of interest (a current list of transgenic mice commercially available worldwide can be found at: http://www.informatics.jax.org/imsr/index.jsp). Second generation transgenic animals are generated using specific promoters (8) or a knock-in approach expressing fluorescence only in a subset of neurons. For example, in transgenic mice expressing enhanced GFP-GAD67, gamma-aminobutyric acid (GABA) interneurons are selectively stained (9). Genetic labelling can also be used to facilitate patch-clamp recording *in vivo* when the pipette is counterstained with a dye that has a different emission spectrum (10).

Even though 2PLSM is still a technique in development (see section 8), significant strides in neuroscience have been achieved using 2PLSM. Here, we review the application of 2PLSM in *in vitro* preparations such as organotypic slices culture and acute brain slices. We will also discuss the imaging of neurons *in vivo* in anesthetized animals. In fact, fluorescence in neurons have been visualized in anesthetized animals up to 500 µm below the pial surface (11, 12), and emerging miniature scan heads may soon become an important tool for imaging cells in freely moving animals (13).

3. FUNCTIONAL IMAGING STUDIES IN SINGLE NEURONS

3.1. Motility of dendritic and axonal protrusions

Dendritic spines, discovered more than 100 years ago by Ramon y Cajal, are tiny membranous compartments consisting of a head (volume ~0.01-1 μm^3) connected to the parent dendrite via a thin (diameter~0.1 µm) spine neck (14). Spines constitute the receiving parts of glutamatergic synapses and are thought to undergo plastic changes during development but also during learning and memory formation. Thanks to the minimal phototoxicity of 2PLSM, time-lapse high-spatial resolution images now allow us to experimentally address this central hypothesis in neurobiology.

The majority of *in vitro* studies support the idea that spines are highly mobile and can change shape within minutes or even seconds. Filopodias in particular appear as ever moving structures exploring new synaptic contacts. Thus, high basal rate of spine motility independent of synaptic activity has been described in organotypic cultures of cerebellum, cortical neurons, and hippocampus (15). This motility was actin-dependent and may play a role in synaptogenesis in young animals (see also section 4.2). These results fit the hypothesis that spine motility subserve the exploration of the environment in the search for an axon terminal (16).

Learning and memory have been associated with synaptic changes occurring during long-term potentiation (LTP) and long-term depression (LTD). While electrophysiological studies have been instrumental in unravel the molecular underpinnings of LTP and LTD, concomitant changes in the morphology of synapses have been predicted since the Hebb's postulate. Several groups have now used 2PLSM in hippocampal organotypic slice culture to directly image dendritic morphogenesis induced by synaptic activity leading to N-methyl-D-aspartate receptor (NMDAR) activation (17-19). The first study (17) used a tetanic stimulus next to a target dendrite (~30 µm) of a neuron infected by an EGFP-Sindbis virus. Only spines close to the stimulus location showed an increase in filopodial protrusions that at times stabilized into a mature spine. A second study (18) applied a tetanic stimulation at the Schaffer collaterals combined with local superfusion of Ca^{++}-containing Ringers of a given population of spines only to limit synaptic transmission to the synapses imaged. 2PLSM was then used for the first on-line demonstration of the birth of a spine triggered by an LTP induction protocol and associated with an increase of synaptic efficacy. Remarkably, such newly formed spines remained stable over 25 hours.

In vitro preparations may lack intact neural circuits that result in artifactual motility. Therefore, *in vivo* imaging of dendritic protrusions has only been possible since the development of 2PLSM. Two pioneering *in vivo* studies observed spine motility of developing (20) and adult (21) barrel cortex. The first study monitored changes in filopodia and spine in layer 2/3 neocortical pyramidal neurons that were transfected by Sindbis virus containing EGFP (20). This study revealed that protrusive motility was experience-dependent in the young rat (P8-P16) and only occurred during the critical period (P11-P13) (figure 1). The second study (21) was conducted in layer 5 neocortical neurons of mice expressing EGFP driven by *thy1* promoter in a small subpopulation of neurons (8). These neurons showed less spine formation and motility compared to the

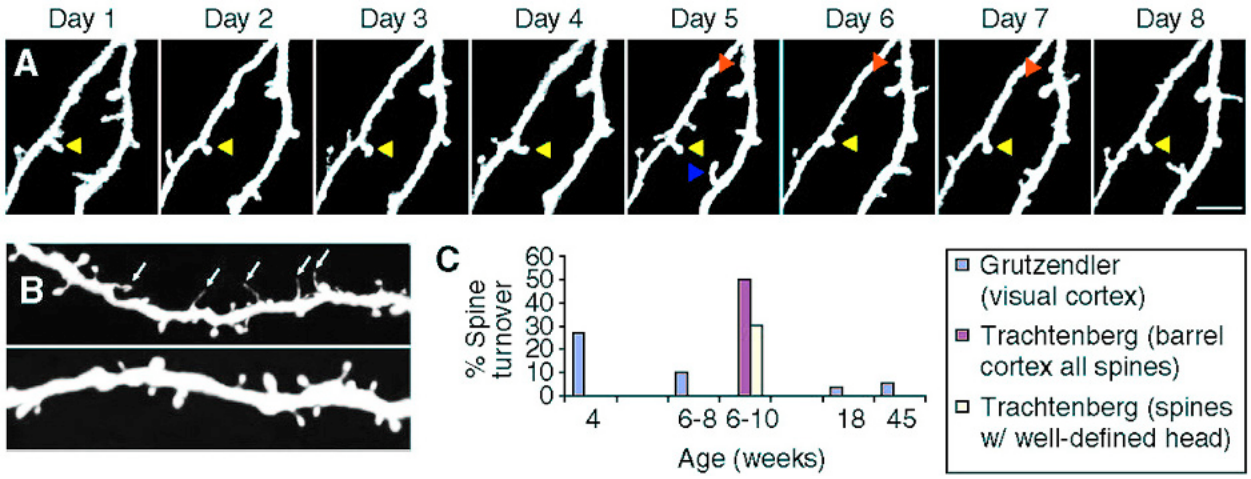

Figure 1. Long-term *in vivo* imaging of dendrites in rodent barrel and visual cortex. A: *In vivo* time-lapse imaging of dendritic segment in rat barrel cortex acquired over eight sequential days. Examples of transient, semi-stable, and stable spines indicated with blue, red, and yellow arrowheads respectively. Scale bar, 5 µm (21). B: *In vivo* imaging of dendritic segment in visual cortex showing abundance of filopodia indicated by arrows, in a 3 weeks old mouse (top panel), and their absence in a 4 month old mouse (bottom panel) (23). C: Summary graph showing fraction of spines turnover in function of age from both groups. Reproduced (with permission) from Meyer *et al.* 2003 (26).

study mentioned above. In both cases, the motility was much less compared with *in vitro* studies. In particular, the rapid movement described in neurons at the surface of organotypic slice cultures (22) was absent and no significant changes were seen within 4 h. Images of an identified single neuron were repeatedly taken for up to several months and they show apparition of new spines within two days. These spines bore typical postsynaptic specializations, such the protein PSD 95, as confirmed by EM. In contrast, a similar study in layer 5 pyramidal neurons of the visual cortex showed remarkably stable spines over 6 months (23). When monitoring dendritic motility at different ages, highly motile filopodias were abundant only in young mice (1 month old) and only in a small proportion of cells. The biological significance and differences between these two divergent results have been greatly debated. (24-26). Regional differences rather than technical issues in the use of the 2PLSM seem to be at the origin of the discrepancy. Indeed, plasticity may be more important in the adult rodent barrel cortex than in the visual cortex (figure 2).

Consistent with *in vitro* studies, *in vivo* studies also show that dendritic filopodia were more active during periods of synaptogenesis. Moreover, mushroom spines typically made stable synaptic contacts but at times also underwent changes in shape. It has been speculated that the change in spine morphology could provide a basis for short-term storage information while new synapse formation may act as a structural basis for long-term information storage. Recently, a study supported this theory and showed that spine enlargement induced by LTP (1 Hz glutamate release in absence of Mg^{++}) was associated with an increase of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropioic acid receptor (AMPAR) mediated currents (27). In contrast, another study using *in vivo* 2PLSM in the olfactory bulb showed perfect dendritic stability over weeks in adult mice expressing yellow fluorescent protein (YFP) even in odor-learning conditions (28). Olfactory sensory neurons (OSN) in the nasal epithelia connecting mitral/tufted cell in glomeruli constantly regenerate throughout life. That makes this an ideal system to study adult neurogenesis. Consistent with this observation, it has been shown that axonal convergence of OSN to glomeruli was extremely precise also in adulthood (29). Thus, these results reinforce the idea that structural plasticity of dendrites may not be required for long-term memory storage.

Most studies focusing on dendritic spine motility and formation do not provide images of the presynaptic counterparts. Since projecting axons are thinner and usually very remote from the soma, dye filling is very challenging if not impossible. Two approaches have been used to overcome this limitation. First, in several studies 2PLSM imaging was followed by electron microscopy (EM) of the very same structure. To this end, fluorescent dyes were either photo-converted (30-32), or GFP-stained neurons were revealed by immunolabelling (33). The latter approach confirmed that dendritic spines exhibited morphological rearrangement even when contacting presynaptic terminals. In addition, the study showed that there was no correlation between spine motility and the extracellular space around individual spines. Coupling EM and 2PLSM of presynaptic elements also allowed for the visualization of rapid axonal motility in hippocampal cultured slices transfected with EGFP (34). Filopodias emerging from mossy fibers terminals were measured from 15-60 minutes and although the resolution of 2PLSM was not sufficient to confirm synaptic contacts, further immunostaining against PSD95 suggested that motile axonal filopodia were immobilized when forming a synaptic contact. This study proposed a solution to the controversy of whether synaptic activity regulated the motility of protrusions. The authors suggested that the motility of axonal filopodia and dendritic spines might be regulated by glutamate in a dose-dependent manner.

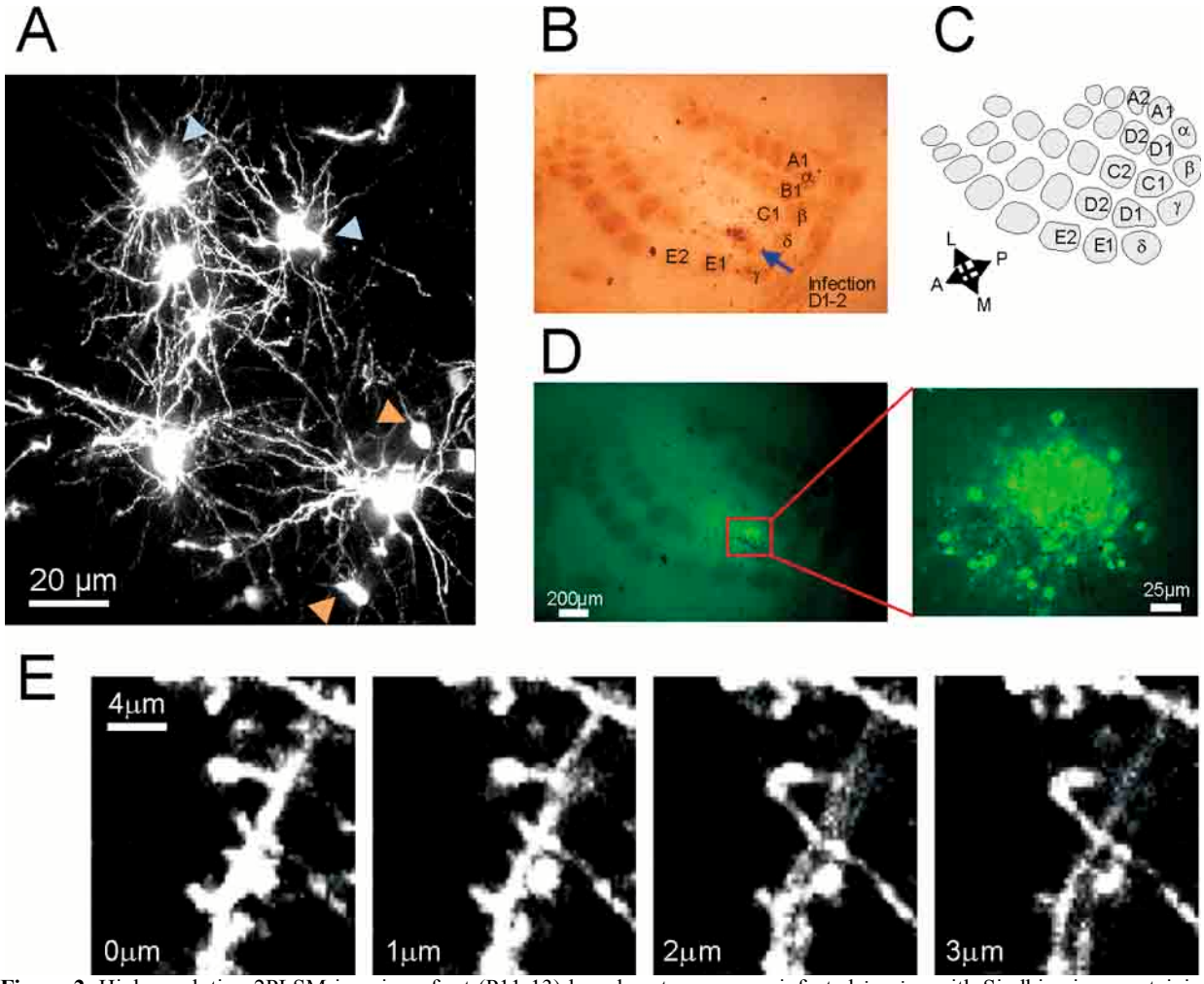

Figure 2. High-resolution 2PLSM imaging of rat (P11-13) barrel cortex neurons infected *in vivo* with Sindbis virus containing the gene for EGFP. A: 2PLSM image of cluster of infected layer 2 neurons (blue arrow heads) with basal dendrites (right panel). Note the cross-sections of thick apical dendrites belonging to deep pyramidal cells (orange arrowheads). B: Histological analysis of the injection site. Brightfield image of layer 4 tangential flattened section stained for cytochrome oxydase (thickness 100 µm) showing the arrangement of the barrel field. Infected neurons appear as dark spots (arrow). C: Schematic representation of the barrel cortex field. D: Fluorescence image of B and an enlargement of the section showing infected neurons. E: High-resolution 2PLSM images showing apparent contact between a spine and an axon. Four optical sections separated by 1 µm are shown. Reproduced (with permission) from Lendvai *et al*. 2000 (20).

Theoretically, an alternate approach to simultaneously monitor pre- and postsynaptic elements would combine genetic labelling with dye filling through a patch pipette. Bichromatic images would be obtained with two fluorophores that were excited at the same wavelength but have a distinct emission spectra. To date, this technique has not been extensively used.

3.2. Calcium and sodium signaling in neurons

Intracellular Ca^{++} is one of the most important second messengers in neurons. For example, at the presynaptic terminal, Ca^{++} controls transmitter release and several forms of short-term plasticity. Postsynaptic Ca^{++} contributes to a multitude of signalling cascade including long-lasting synaptic plasticity, such as NMDAR-dependent LTP. The intracellular calcium concentration ($[Ca^{++}]$) is therefore precisely controlled in time and space. 2PLSM can be used to image $[Ca^{++}]$ transients with high spatial (<1 µm) and temporal resolution (~2 ms), to study diffusion properties of Ca^{++} (FRAP, see section 6), and to focally manipulate Ca^{++} concentrations (2P photolysis, see section 5).

Increase of $[Ca^{++}]$ in small compartments such as dendritic spines can be measured by 2PLSM. Absolute $[Ca^{++}]$ transients are best measured using ratiometric Ca^{++} imaging (35). Since changing the wavelength in the tunable 2P lasers takes several seconds, $[Ca^{++}]$ changes that occur within milliseconds cannot be measured ratiometrically with a single laser. As an alternative, $[Ca^{++}]$ can be estimated by taking into account the maximal fluorescence obtained at dye saturation (36). With this approach, $[Ca^{++}]$ transients in spines evoked by a single action potential (AP) were found to be very large (up to 1.5 µM) and very brief

(~12 ms, which is ~100 fold faster than the dissipation of Ca^{++} by diffusion through the spine neck) (37). These results suggested that the Ca^{++} in the spines was subject to active extrusion. In addition fluctuation analysis (see also (38)) has been used to measure diffusional coupling between spine heads and dendrites (37) as an alternative to FRAP experiments (see section 6) that have been shown to provoke photodamage which retards diffusion in neurons. 2PLSM also revealed that even aspiny dendrites of neocortical interneurons possessed Ca^{++} microdomains (39). They were generated by a low and transient Ca^{++} entry through AMPAR that lack the GluR2 subunit and were spatially limited by the action of endogenous immobile Ca^{++} buffers and Ca^{++}/Na^{+} pumps. The measured microdomain size was at the limit of the 2PLSM resolution, suggesting that with a mobile Ca^{++} indicator (here Fluo-4), the size of Ca^{++} microdomains (approx 0.3-0.5 µm) may have been overestimated.

The above-described compartments receive Ca^{++} mainly through voltage-gated calcium channels (VGCC), NMDAR and intracellular stores. 2PLSM now allows for direct visualization of these sources. For example, combining 2PLSM with whole-cell patch-clamp recordings in CA1 neurons showed how the selective inhibition of R-type VGCC in spines blocked the induction of LTP (40). With the low affinity indicator Fluo-5F, the changes of free Ca^{++} concentration produced by a single AP were monitored and trial to trial fluctuation analysis was then used to estimate the number of VGCC involved (38).

Studies on NMDAR have been performed to measure the contribution of these receptors to synaptic transmission in hippocampal CA1-CA3 synapse. Previously, electrophysiology techniques, such as electrical minimal stimulation, were used to measure quantal neurotransmitter release, but it remained impossible to identify the active synapse. 2PLSM line-scan mode in CA1 neurons showed that NMDAR $[Ca^{++}]$ transients evoked by minimal stimulation could be used as detector of quantal release of neurotransmitter (41). Moreover, the signal-to noise ratio for imaging of Ca^{++} transient was better than with electrophysiological recordings in the soma. This study concluded that NMDAR in single spines were not saturated. This result was surprising because NMDARs have a high affinity for glutamate and the number of NMDAR was much smaller than the number of glutamate molecules released (42).

2PLSM has also been used to monitor $[Ca^{++}]$ transients in presynaptic terminals. For example, presynaptic modulation by Ca^{++} stores changed the inhibitory mPSC (mIPSC) amplitude in an interneuron-Purkinje cell synapse (43). This study measured presynaptic $[Ca^{++}]$ in response to short trains of APs and concluded that large amplitude mIPSCs were multivesicular events, which introduced an amplification step in the control of neurotransmitter release.

2PLSM was also used to measure Ca^{++} dynamics in dendrites *in vivo*. In neocortical pyramidal neurons of layer 2/3, the authors were able to record intracellular somatic currents and perform calcium-green 1 imaging down to 500 µm below the pial surface (11). Dendritic Ca^{++} influx was carried by VGCC activated by dendritic Na^{+} AP and its amplitude decreased with increasing distance from the soma. In contrast to an *in vitro* study (44), subthresold depolarization did not elicit Ca^{++} influx *in vivo*. Layer 5 neocortical neurons (12) located deeper than the present 2PLSM detection limit were loaded dendritically or somatically by a sharp electrode with calcium-green 1. Dendrites were then visualized in the first 500 µm below the surface. In contrast to layer 2/3 pyramidal neurons, dendritic Ca^{++} transients elicited by Na^{+} AP were detectable over 100 µm away from the soma. Moreover, dendritic Ca^{++} electrogenesis happened in conjunction of Na^{+} AP burst firing, but could not be detected with a single AP. These experiments investigating the coupling between dendrite and soma in response to APs will be possible in conscious animals due to development of miniaturized 2PLSM (13) and facilitated by transgenic GFP calcium indicators mice (45).

Until recently, the existence of variations in extracellular $[Ca^{++}]$ in the neuropil remained elusive. This is because such changes were very difficult to measure given that all known Ca^{++}-indicators are saturated at extracellular $[Ca^{++}]$ of approx. 2.5 mM. This limitation could be overcome with a very low affinity Ca^{++}-indicator (ex. Rhod-5N, K_d= 320 µM) delivered locally to the extracellular space via a pressurized patch pipette (46). With this approach the authors measured a small but significant decrease in extracellular $[Ca^{++}]$ after just five extracellular electric stimuli (100 Hz) 50-100 µM below the surface of an acute slice of the stratum radiatum in CA1. With this technique, it was not possible to monitor a single synaptic cleft, which was below the optical resolution of laser-scanning microscopy. The extracellular changes of $[Ca^{++}]$ of 1% representing about 40-50 µM could be interpreted in two ways: first, as an almost total depletion of extracellular Ca^{++} at all active synaptic clefts if one considered the small fraction of synaptic cleft volume versus the tissue volume; second, as a more moderate and/or heterogeneous depletion over a larger volume.

Finally, an elegant study combined Ca^{++} measurements in dendrites of retinal starbust amacrine cells (47) with patch-clamp recordings in response to light stimulation of photoreceptors. This was possible because the infrared light used for 2P excitation did not activate photoreceptors of the retina. Dendrites of starbust amacrine cell responded to light movement with Ca^{++} transients only when light stimuli moved centrifugally from the cell soma.

The techniques and experiments used to associate a function for Ca^{++} variations in synaptic physiology were also applicable to Na^{+} imaging (48, 49) (see also chapter by J. Kockskämper *et al*.). Sodium-binding benzofuran isophthalate (SBFI) is a ratiometric Na^{+} indicator originally developed for 1P excitation. This compound can be readily excited at 790 nm with a 2P light source to obtain non-ratiometric read-outs. In hippocampal acute slices massive Na^{+} entry into dendrites and spines (up to 100 mM) was observed with synaptic stimulation. Again, the high spatial

resolution of 2PLSM allowed for identification of large Na^+ microdomains in the dendrite (<30 μm) that are probably maintained by pumps and buffers (49).

3.3. Receptor trafficking and synaptic plasticity

Over the last years a number of studies have indicated that many neurotransmitter receptors rapidly redistribute with synaptic activity, which may be a mechanism underlying experience-dependent synaptic plasticity. For example, rapid insertion of AMPARs has emerged as one of the main mechanism of LTP expression. This is supported by imaging evidence from cultured cells and electrophysiological observations in slice preparations (50). 2PLSM now allows for a direct visualization of the mobile AMPARs. For example, one study using organotypic slice cultures showed that after LTP induction to Schaffer collaterals, a rapid delivery of GFP-tagged GluR1 occurred in spines and clustered in the dendrites of CA1 neurons (51). These changes happened within 30 min and were dependent on NMDAR, which further supports the hypothesis of rapid AMPAR insertion underlying LTP (52). Additional studies using GFP constructs of GluR1 mutants expressed in hippocampal organotypic slices confirmed the subunit-specific localisation and movement in response to synaptic activity. Furthermore, visualization of GFP-GluR2 and GFP-GluR3 constructs confirmed the interaction of these subunits with N-ethylmaleimide-sensitive factor (NSF) for their constitutive delivery to spines (53). In a technical tour de force, dual-channel 2PLSM imaging was used to simultaneously visualize the neuron filled with Texas Red as well as the transfected mutated GFP-AMPAR subunits (54).

Finally, visualisation of GFP constructs of the small GTPases, Ras and Rap, have been useful in determining their implication in AMPAR trafficking during LTP and LTD (55). In this study, the AMPAR subunit was identified using an electrophysiology tag instead of 2PLSM. Ras has been shown to be involved in synaptic delivery of AMPAR during LTP. In contrast, Rap was responsible for synaptic AMPAR removal during LTD.

Photobleaching experiments are an alternative to follow GFP tagged receptors (see section 6). A promising approach is to develop transgenic mice with photoactivable GFPs tagged receptors. This method may help to overcome overexpression artefacts due to transfection. Another method to characterize membrane receptor movement consists of immunolabeling the receptor with fluorescent beads (56). This technique has the disadvantage to label many receptors because of the large size of the bead and the poor penetration in the slice preparation. Finally, in dissociated neurons single receptor resolution has been reached by using antibody coated fluorescent quantum dots (see section 8).

4. FUNCTIONAL IMAGING STUDIES IN NEURAL NETWORKS

4.1. Mapping brain activity

How neuronal networks accomplish information processing is central for the understanding of higher brain function. Until recently the investigation of functional neuronal networks have been limited to large-scale brain imaging techniques with rather low resolution (e.g. EEG or functional magnetic resonance imaging (fMRI)). Spontaneous network activity as well as stimulus-evoked activity of small networks can now be monitored with 2PLSM after loading many cells at the same time with cell-permeant Ca^{++} indicators (AM-esters).

Fura2-AM loading was successful in newborn rats cortical slices (P0-12) and was used to map long-range neuronal connection between neurons (1) (figure 3). Up to 1400 neurons of the visual cortex in acute slices of mouse (P14-P21) were monitored to reconstruct the spatiotemporal dynamics of cortical spontaneous activity (2). In older animals, bulk AM-loading yielded images of lesser contrast. Thus, this method seems restricted to experiments performed in young animals. However, imaging neuronal network activity *in vivo* or in a slice has become possible by using the multicell bolus loading technique (MCBL). Local ejection of AM-ester dye into a slice can stain several hundreds of cells within one hour of loading, and reach a depth of ~200 μm (3).

Visualizing neuronal network activity in response to specific stimuli has been achieved in the fruit fly (57-59). The first study dissected the transmission of olfactory stimuli in the antennal lobe of *Drosophila Melanogaster* using synaptopHluorin, a membrane-bound pH-sensitive dye (57). This GFP-based probe acted as a reporter of synaptic activity allowing the researcher to image cycling of synaptic vesicles. SynaptopHluorin has been expressed in three different populations of neurons and their respective synaptic terminals were visualized with 2PLSM. Odor-specific activation of a subset of neurons provoked an increase of fluorescence at their synaptic terminals, which represented neurotransmitter release. Thus, a map of odor-specific activity was characterized in the glomeruli of the olfactory bulb. A second study used the same paradigm with other odor-stimuli and the transgenic expression of a Ca^{++}-sensitive GFP (45) dye in olfactory sensory neurons and glomerular projection neurons (58). Upon activation, Ca^{++} increases were monitored in targeted neurons, and a map of activity was established.

Neuronal network activity can also be followed by fluorescence of voltage-sensitive dyes. However, this method has not yet been used with 2PLSM because the dyes available are not optimal for fast membrane potential changes and the fractional changes of fluorescence are small resulting in a low signal to noise ratio. However, development in voltage-sensitive dyes for 2PLSM may render them suitable for such applications in the near future (60, 61) (see also chapter by B. Kuhn).

Finally, blood flow measurement is an indirect method to measure neuronal activity. An increase in the blood flow is concomitant with neuronal activation in a particular area. By injecting a fluorophore such as fluorescein dextran in the blood, 2PLSM has been used to image *in vivo* the motion of counterstained red blood cells

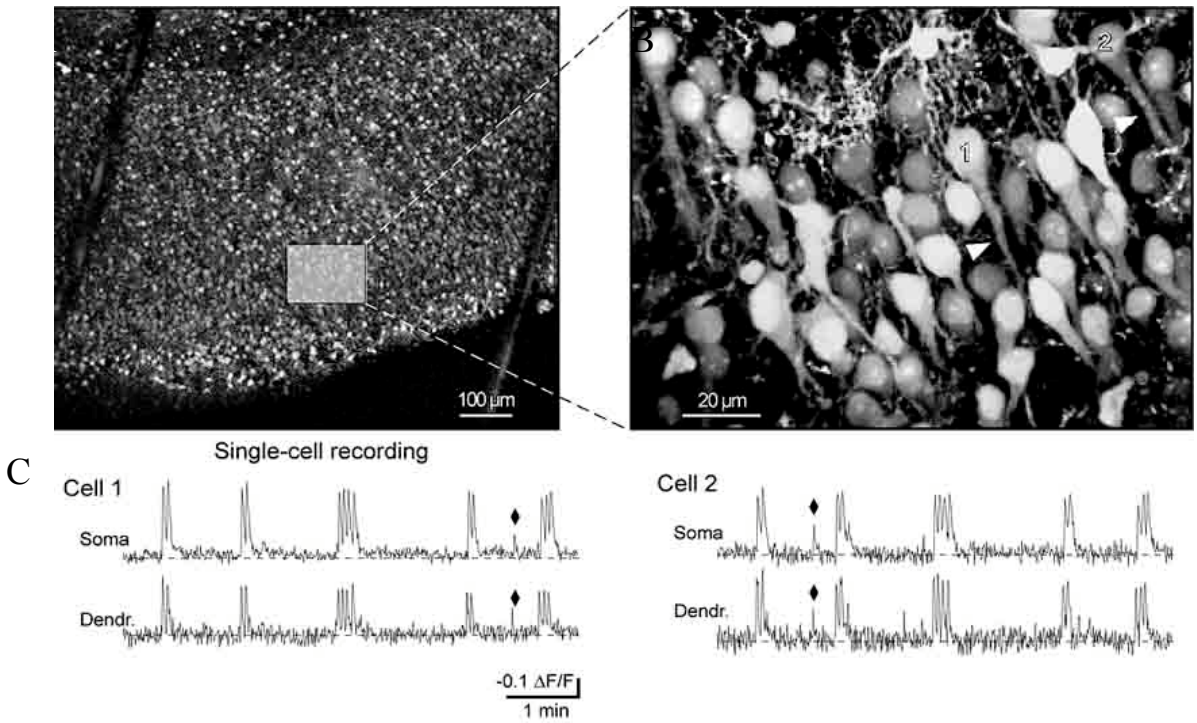

Figure 3. Network Ca^{++} oscillations recorded in rat temporal cortex. A: 2PLSM image from a temporal cortex slice of a 3 days old rat loaded with fura2-AM. The region in the shaded box is shown in (B) at a higher magnification. B: High-resolution 2PLSM image layer 2/3 showing individual neurons of and their projections. Cell 1 and 2 Ca^{++} measurements are shown in (C). Arrowheads indicate where dendritic line-scan recording has been performed. C: Somatic and dendritic Ca^{++} recordings (ΔF/F) of the two cells marked in (B). Majority of Ca^{++} waves are synchronous. Diamonds indicate asynchronous Ca^{++} transients. Reproduced (with permission) from Garaschuk *et al*. 2000 (1).

in response to an external stimulus. One study described blood flow variations up to 600 µm below the pial surface in the primary somatosensory cortex in response to whisker stimulation or cutaneous stimulation in rat (62). When the velocity of red blood cells increased, their shape elongated. Changes in blood flow in response to an odorant stimulus were also visualized in glomerular capillaries of the olfactory bulb *in vivo* (63). Loading in capillaries by quantum dots has been possible and gave a qualitatively similar result (64) (see section 8.4).

4.2. 2P imaging of developing brain

2PLSM has been used to visualize the development of single neurons, (65) axon path finding (29), cell migration (66), and dendritic remodelling (15, 16, 67-70) (for review see (71)).

The very first study, which used 2PLSM to follow cellular development over a 24 h period, was carried out in mitotracker-labeled hamster embryos. Time-lapse imaging showed the change of mitochondrial distribution occurring during the second and third cell division (72). In addition, an *in vivo* study used time-lapse imaging for over ten hours to examine retinal progenitor cell division during neurogenesis in the zebrafish (65).

Tau-GFP linked to olfactory receptors in mice has been used to show axonal convergence of OSN to glomeruli. A head-mounted preparation was performed for imaging from 10 min to 4 h after dissection. 2PLSM in section series permitted single axon detection at the level of the olfactory epithelium. Moreover, high resolution imaging in the olfactory bulb of glomeruli was achieved, where glomeruli were ~80 µm big and reached a depth of ~200 µm from the surface (29).

Migration and settling patterns of GABAergic interneurons originating in the ganglionic eminence of the ventral forebrain, and incorporation into the neocortex of the cerebral hemispheres of mice has been monitored by time-lapse 2PLSM for up to 24 h depending on the preparation (66). This study is the first documented *in utero* imaging of the viable developing neocortex.

Soon after birth, the relatively smooth dendrites of neonates sprout numerous filopodia-like protrusions, which are later replaced by dendritic spines as the brain matures. This was monitored in the retina, which was isolated as an intact sheet of tissue without disrupting its structure and function and it remained viable fore more than 36 h permitting gene transfection (67). Dendrites of GFP transfected ganglion cells showed highly motile filopodias. Surprisingly, this dynamic dendritic remodelling resulted in a total change in length equal to zero. Blockade of glutamatergic transmission at E13-16 in chicken suppressed dendritic motility and decreased both the total

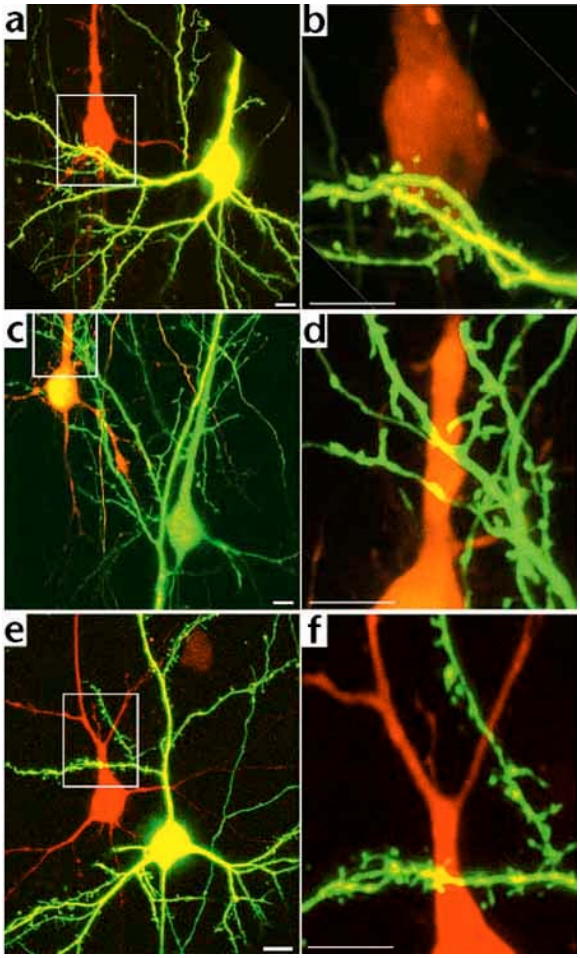

Figure 4. Dual-channel 2PLSM imaging showing the influence of BDNF in dendritic complexity. A and C: Dendritic branching increases when GFP-recipient neurons are in close proximity to RFP-BDNF donor neuron. Boxes enlarged in B and D, respectively. E: Control neurons expressing only RFP did not increase dendritic complexity in GFP-recipient neurons. F: High-magnification of neuron E. Scale bars 10 µm. Reproduced (with permission) from Horch and Katz 2002 (69).

dendritic length and the number of branch points in the dendritic tree. This result suggested that dendritic remodelling promoted synaptogenesis, because expanded volume increased the probability that an afferent would contact its synaptic partner.

Neurotrophic factors have also been shown to modulate dendritic organisation. For example, BDNF overexpression was shown to increase the number of basal dendrites on neurons (68). The authors developed a strategy to precisely monitor the role of BDNF in dendritic branching. Their idea was to biolisticaly transfect one set of neurons with an RFP-BDNF construct (donor neuron) and another set with only GFP (recipient neuron). Thus, in the visual cortex, dendrites of recipient neurons were affected depending on the proximity to the soma or dendrite of a BDNF donor neuron (69). Thus, 2PLSM revealed increases in dendritic branching near the source of BDNF, without effects of BDNF on axons or spines (figure 4).

5. 2-PHOTON-PHOTOLYSIS (2PP)

Theoretically, pulsed IR lasers can also be used for 2PP of caged compounds with similar advantages as 2P-imaging: less light scattering, and therefore a deeper penetration and a very focal uncaging area. This is quite different from "chemical two photon uncaging" where out of focus photolysis is reduced by using compounds with two caging groups (73, 74). One of the first studies used 2PP in cardiac myocytes, but the same approach is also applicable in neurons (figure 5). 2PP, however, is still not widely used. This is mainly because compounds designed for 1P photolysis such DM-nitrophen have a very low cross-section for 2P excitation at wavelengths near IR (<0.1 GM) (75) (see also chapter by G.C. Ellis-Davies *et al*.). Newer compounds, such as the Ca^{++} complex azid-1 (76), which has a cross-section of 1.4 GM were synthesized to solve this problem.

A different caging group, brominated 7-hydroxycoumarin-4-ylmethyls (bhc, GM=1 at 740nm (75), has also been used with 2PP but has very slow uncaging kinetics. Caged glutamate (bhc-glu) was synthetized and tested in visual cortex brain slices (P12-19, 400 µm). Bhc-Glu 0.5 mM was perfused from a capillary tube located next to a patch-clamped cell. The onset of glutamate response with 2P uncaging was slow and the amplitude was small compared to MNI-glu (described below). Thus, to get a reasonable response, a higher power of the laser was needed (up to 75mW, during 10 ms versus 3-7 mW, 2 ms for MNI-Glu (27, 77)).

Although MNI-glutamate has a rather low cross-section (0.06 GM) it has been successfully used with 2PP to map functional glutamate receptors at the level of a single synapse in CA1 neurons (77). The results indicated that distribution of functional AMPAR was tightly correlated with spine geometry and that receptor activity was independently regulated at the level of a single synapse. In another study repetitive MNI-glutamate uncaging induced a rapid and selective enlargement of stimulated spine that is transient in large spines (>0.1 μm^3) and persisted up to 100 min in small spines in slice cultures of EGFP-CA1 pyramidal neurons (27). Spine enlargement was associated with an increase of AMPAR mediated currents (perforated patch) at the stimulated synapse and was dependent upon NMDAR, CAMKII, calmodulin activation, and actin polymerisation but not on metabotropic glutamate receptor (mGluR) activation. Taken together, these studies show that both structural and functional plasticity can be induced at the level of an individual spine using 2P photolysis of caged glutamate.

Finally, one study used Gamma-ANB-Glutamate 1P photolysis in combination with 2PLSM to monitor Ca^{++} influx in dendritic spines (250 µM calcium-green 1(78)). When glutamate was paired with synaptic AP, Ca^{++} influx through the NMDAR channel was selectively amplified about 3 times along ~ 6.5 µm of the imaged dendrite. This technique, however, had a lower resolution than required to

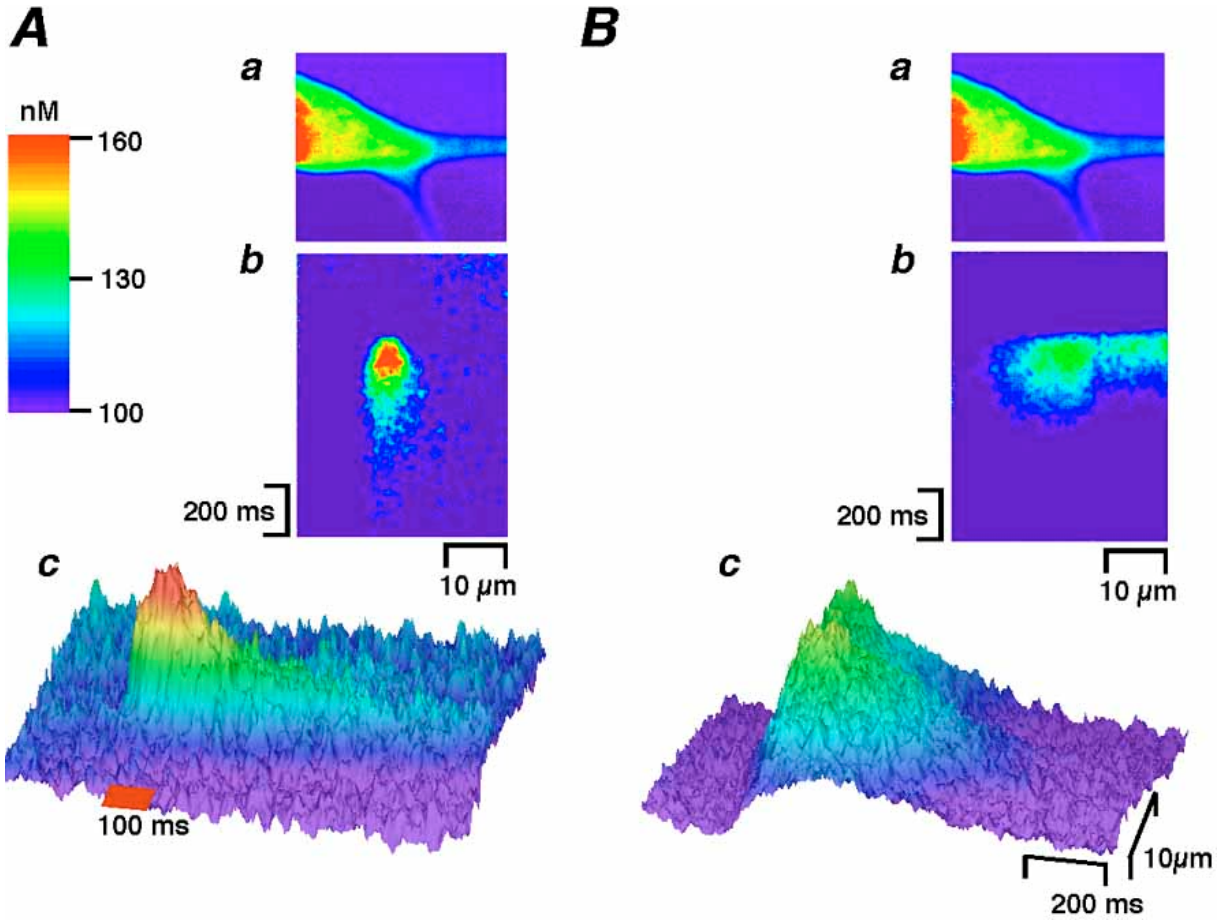

Figure 5. 2PP of DM-nitrophen in dissociated culture of rat hippocampal neurons. A: A short 2PP exposure of a cultured hippocampal neuron held at –70 mV (a) leads to a spatially restricted artificial Ca^{++} spark (ACS) peaking at ~150 nM, which dissipates within a few hundred milliseconds, extending spatially not more than 20 µm. (b) shows the line scan image, (c) the 3D surface plot of the line scan of the ACS. B: A Ca^{++} transient elicited by a single action potential reaches similar Ca^{++} concentrations, but is spatially less confined as the action potential propagates into the adjacent dendrite. (b) The line scan image, (c) The 3D surface plot of the line scan of the Ca^{++} transient. The intracellular solution contained (in mM): K-Gluconate 120, KCl 20, K-ATP 4, Fluo-3 0.1, CaCl2 0.25, DM-nitrophen 1. The superfusate consisted of (in mM): NaCl 150, KCl 4, glucose 30, CaCl2 2, MgCl2 2, HEPES 5, Na-pyruvate 2, and - in order to reduce spontaneous activity, which could lead to Ca^{++} entry, TTX 1,5 µM, bicuculline 20 µM, strychnine 5 µM and amino-5-phosphopentatonic acid (APV) 50 µM.

activate a single synapse (>1 µm). In the future more studies will have to combine 2PP with 2PLSM (27), which requires two pulsed IR lasers, an acquisition that until recently was prohibitively expensive for most labs.

6. FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING (FRAP)

The ability to excite mobile fluorophores in a very small, confined volume has been used to monitor 'fluorescence recovery after photobleaching' (FRAP) in order to demonstrate diffusion properties between cellular compartments. Diffusion could also be estimated by measuring photo-released caged fluorophore. To date, FRAP experiments have been performed to investigate the compartmentalisation of diffusible molecules such as Ca^{++}. Several studies have focused on the Ca^{++} diffusion between dendrites and spines, because postsynaptic Ca^{++} can trigger various forms of synaptic plasticity.

An early study combined 2PLSM and FRAP using fluroescein dextran to measure diffusion between spine head and dendritic shaft (79). The initial hypothesis was that the spine head functions as a chemical compartment and the geometry of the neck controls spine synaptic weight. With 2P excitation the required spatial confinement of photobleaching was achieved. To image the fluorophore, a second 2P laser source was used, which showed that the time constant for diffusional relaxation of the spine head was dependent on neck length. However, changes in spine neck geometry would not have significant role in controlling synaptic weight if $[Ca^{++}]$ decreased dramatically before reaching the dendrite. Thus, not only the morphology of the spine neck but also active extrusion by Ca^{++} pumps would contribute to the kinetics of Ca^{++} transients in the spine. Indeed, two classes of spines have been characterized: in some spines, the Ca^{++} pumps dominated, whereas in other spines the diffusion of Ca^{++} played a major role (80). This study used Pockels cells to

rapidly modulate laser intensity in order to switch between photobleaching and the visualization mode (81). A conclusion of this study was that changes in spine neck length had an impact on Ca^{++} decay. Thus, spines may continuously change their capacity for synaptic plasticity (82). However, this approach was limited by the high concentration of high affinity Ca^{++} dyes that may have altered 'real' Ca^{++} diffusion properties (38).

As a matter of fact, FRAP may not be the perfect substitute for Ca^{++} flux measurement, but may be more appropriate to monitor protein dynamics. To date, most of these studies have been performed with epifluorescence or 1PLSM (reviewed in (83)). For example, EGFP-actin turnover in dendritic spines has been monitored with 1PLSM (84) and showed that NMDAR-dependent LTD stabilized actin filament, which led to a reduction of spine motility in culture of hippocampal dissociated neurons.

7. MEMBRANE-BOUND FLUOROPHORES (FM dyes)

FM 1-43 is a member of a family of water-soluble stiryl dyes that bind to the cell membrane with low affinity (reviewed in (85)). It can therefore easily be washed from the preparation unless the stained membrane has been endocytosed. FM 1-43 has been extensively used in dissociated cultures to monitor the presynaptic dynamics of vesicles with synaptic activity. FM 1-43 was loaded into the presynaptic terminals using electrical stimulation of the synapse to promote vesicle recycling. Many studies have also used the frog neuromuscular junction with a combination of conventional epifluorescence microscopy, electron microscopy and electrophysiology (86). Recently a scavenging compound, cyclodextran (beta-cyclodextrin sulfobutyl ether, 7 sodium salt) has become available that allows the rapid washout of stiryl dyes in the neuromuscular junction (87), but also acute brain slices and organotypic cultures (88). Thus, FM dyes have been used to elucidate mechanism of various forms of synaptic plasticity. For example, CA3 - CA1 excitatory synapses in hippocampal slices show a reduced "readily releasable pool" of vesicles in association with a presynaptic form of LTD (89). In younger rats, mGluR dependent LTD was also associated to a reduction of presynaptic release as assessed with 2PLSM of FM 1-43 (90). Using 2P imaging of FM1-43 in acute slices of BDNF -/- mice, arguments have been found for a presynaptic BDNF dependent component of LTP in the hippocampus (91). FM dye was ejected by a pipette into CA1 stratum radiatum at a depth of 100-150 µm and a stimulus train applied to Schaffer collaterals was used to load the dye. The unloading rates were measured before and after LTP induction.

8. EMERGING DEVELOPMENTS

In the previous sections we have outlined a myriad of new possibilities offered by 2PLSM and given examples of how this technique can be used for the benefit of neuroscience. However, there are limitations that still preclude the use of 2PLSM in some situations. To overcome these obstacles, improvements in the hardware as well as in the dyes will have to be made.

8.1. Make 2PLSM faster

Currently, fast acquisition is only possible at the expense of spatial resolution. For example, measurements of fast events like Ca^{++} kinetics are typically done in line-scanning mode. Alternatively, multifocal microscopy, using a beam splitter or microlenses, has been developed to excite many foci simultaneously (92-95). To reach video-rate acquisition (30 frame/s), the scanner was modified by substituting the x-y galvano-mirror with an acousto-optic deflector (96). However, these modifications also needed additional correction to compensate for light dispersion. Many current systems already allow for acquisition during the returning path of the laser beam, which is not normally used. This has been possible using resonant galvanometers and an adapted acquisition software (97, 98).

8.2. Miniaturize 2PLSM

Size reduction of the scan head is essential for *in vivo* recordings of awake, freely moving animals. Theoretically, the weight of the existing miniature head-mounted 2P microscope could be reduced from 25 g to 5 g including improvement of optical performances (13). In addition these miniature systems could technically be coupled to electrophysiology recordings in head-movement restrained, but awake animals (99).

8.3. Access remote brain regions

Strategies to reach remote brain regions *in vivo* consist of either amplifying laser light up to 1 mm below the surface (100) or using gradient index lenses for microendoscopy (101, 102). To date the hippocampus could only be imaged *in vivo* by 2PLSM after removal of the neocortex (103). With this approach, the recordings were limited by the duration of the anesthesia. In this study, no significant changes in dendritic spines were detected even in the presence of epileptic activity, which was consistent with the rate of spine turnover measured in the barrel or the visual cortex (21, 23). It is possible that the emerging techniques described above will allow for more extensive monitoring.

8.4. Brighter fluorophores

Dyes with less photodamage and photobleaching properties (104) and with higher 2P cross section (105) would increase the possibilities of 2PLSM (see also chapter by Minta *et al*.). For example, quantum dots (QD) with a large 2P cross section and a high quantum efficiency fluorescence, but virtual absence of photobleaching have been developed (106, 107) and are very promising for multiphoton imaging. 2PLSM imaging of QD has been performed *in vivo* in capillaries through high scattering tissue, such as skin and adipose tissue up to 250 µm deep (64). Moreover, QD did not seem to be toxic to living cells since micelle-encapsulated QD have been injected in Xenopus embryos that eventually developed normally (108). QD have the ability to be coated by specific antibodies that have the benefit of targeting fluorescence to individual proteins. Thus, glycine receptor diffusion dynamics have been measured in cultured neurons (109). Hopefully, this technique will be exploited in tissue or *in*

vivo preparation to monitor protein localization and movements in subcellular compartments. Targeted genetic staining is probably the most convenient tool for 2PLSM (110). Until improvement in yields of viral transfection *in vivo*, more transgenic mice have to be developed to monitor Ca^{++}(45, 111), Cl^{-} (112), or pH changes (113). We have described FRAP experiments that measured protein dynamics but photoactivation may lead to new perspectives. New photoactivatable GFP variants increased fluorescence upon activation up to 2000 fold and would allow for monitoring tagged proteins (83, 114, 115).

8.5. Apply established microscopy techniques to 2PLSM

Different established imaging techniques can also be used with 2PLSM, but are currently not frequently used. For example, fluorescence resonance energy transfer (FRET) has been successfully used to image CFP-YFP constructs (116, 117). As FRET would reduce fluorescence intensity and fluorescence lifetime of the donor, fluorescence lifetime microscopy (FLIM) could be use to detect FRET (118) (see also chapter by Niggli and Egger). Total internal reflection fluorescence (TIRF) using inverted-microscope has been developed to monitor principally exo-/endo-cytosis (119, 120). Recently, 2PLSM has been used for histological reconstructions. By increasing the laser power (1-10 nJ pulses), fresh or fixed tissue was sectioned and imaged approx. every 100 µm and reconstructed in 3D (121). Combination of 2PLSM with second harmonic generation microscopy (SHGM) is also a promising technique which can reveal cellular structures (122). Indeed SHGM could image either native or exogenous non-symmetric molecules such microtubules (123) or stiryl dyes (124), respectively. Native fluorescence can also be detected by 2PLSM by 2P excitation for NADH (125) or 3P excitation for serotonin (126) (listing in (127)).

8.6. Monitoring disease

Finally, an emerging application of 2PLSM also includes the monitoring of pathological processes. For example encephalitic T cells causing multiple sclerosis have been followed after staining with rhodamine cell tracker (128). In an animal model of Alzheimer's disease, amyloid plaques were revealed with thioflavine S staining (127, 129, 130) and neurofilament tau (NFT) was detected with SHGM (127). Therefore, in the future 2PLSM may complement functional imaging techniques such as fMRI to image with a high temporal resolution in deep tissue at the cellular level in human.

9. AKNOWLEDGMENTS

We thank Nicolas Demaurex, Mathias De Roo, Cheryl L. Marker, Dominique Müller and Tanya R. Stevens for comments on the manuscript and the classes of the 1999 and 2003 Cold Spring Harbor Laboratory course on "Imaging structure and function in the nervous system".

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Key Words: Fluorescence, GFP, Multi-photon, Infrared Light, Two-Photon Laser Scanning Microscopy, Neuron, Synapse, Network, Neuroscience, Imaging, Calcium, Fluorophores, Photobleaching, Review

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