Experimental Physiology

Dynamic Confocal Imaging of Living Brain

Functional imaging of single synapses in brain slices

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The strength of synaptic connections in the brain is not fixed, but can be modulated by numerous mechanisms. Traditionally, electrophysiology has been used to characterize connections between neurons. Electrophysiology typically reports the activity of populations of synapses, while most mechanisms of plasticity are thought to operate at the level of single synapses. Recently, two-photon laser scanning microscopy has enabled us to perform optical quantal analysis of individual synapses in intact brain tissue. Here we introduce the basic principle of the two-photon microscope and discuss its main differences compared to the confocal microscope. Using calcium imaging in dendritic spines as an example, we explain the advantages of simultaneous dual-dye imaging for quantitative calcium measurements and address two common problems, dye saturation and background fluorescence subtraction. *Experimental Physiology* (2002) **87.6**, 733–736.

Many processes that shape the brain's responses to the environment are thought to operate at the level of individual synapses. The axon of a single pyramidal cell, for example, is able to form connections with very different characteristics depending on the type and the activity of the postsynaptic cell (Markram *et al.* 1998). However, electrophysiology typically reports the summed activity of many synapses. For the study of single synapses *in situ*, functional imaging has proved to be a valuable tool. Most excitatory synapses in the cortex are located on spines, tiny protrusions of the dendrite that act as biochemical compartments. By optically monitoring calcium transients in spines, the local postsynaptic response after activation of a single synapse can be measured (Denk *et al.* 1996; Mainen *et al.* 1999*a*). The amplitude and duration of postsynaptic calcium transients are known to be critical for the induction of long-term plasticity, so by measuring these transients we can tap into the very process that shapes synaptic properties on longer time scales. Since we need to image the same tiny structure over and over again, wasteful excitation of fluorescence, leading to unnecessary photodamage, has to be avoided. For optical calcium measurements in highly scattering brain tissue, the two-photon microscope is the tool of choice.

A two-photon microscope is a laser scanning microscope that uses a long wavelength laser to excite fluorescence (near infrared, 800–1000 nm). At these wavelengths, the energy of a single photon is not sufficient to excite fluorescent dye molecules. It is only if two photons are absorbed at the same time by a dye molecule, that this molecule will reach the excited state and subsequently emit a photon. In contrast to normal one-photon fluorescence, the emitted photon will have a higher energy (i.e. shorter wavelength) than either of the absorbed photons. Because two-photon absorption events are exceedingly rare, the excitation light must be concentrated not only in space by focusing the collimated laser beam, but also in time by using a laser that produces ultra-short light pulses at a high repetition rate. A detailed description of the physical principles relevant to two-photon microscopy can be found in a paper by Denk *et al.* (1995), and practical issues of microscope design are discussed by Denk & Svoboda (1997) and Tsai *et al.* (2002).

What is the advantage of using two-photon absorption to excite fluorescence? The focused laser beam forms a cone of light in which the intensity (i.e. density of photons) falls off with the square of the distance from the focus. The probability of two-photon absorptions is proportional to the square of the density of photons. Taken together, the probability of two-photon absorptions falls off with the 4th power of the distance from the focal plane, in practice limiting fluorescence excitation to a tiny focal volume of about 0.1 μm^3 (for a 1.4 NA objective). This localization of excitation is the key advantage of two-photon microscopy.

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Experimental Physiology: Translation and Integration

Since all emitted photons originate from the focal volume, there is no need for a confocal pinhole to reject out-of-focus fluorescence. Instead, wide-field detection is employed to capture as many emitted photons as possible, even the ones exiting the preparation through the condenser. Localization of excitation also guarantees that undesirable effects of the illumination (bleaching and phototoxicity) will be restricted to the focal volume only.

Using long-wavelength excitation brings about several beneficial side effects. First, the longer wavelength will be less scattered in tissue, allowing for deeper penetration at a given laser intensity. In addition, scattered infrared photons are harmless: they are too dilute to cause any excitation in the tissue. Second, the excitation spectra of most fluorescent dyes are much broader for two-photon excitation, which means that different fluorescent dyes can be excited simultaneously by the same laser beam. As a third advantage, there are no endogenous fluorophores in nerve cells that can be excited by two-photon excitation at this wavelength, leading to extremely low levels of auto-fluorescence. If background fluorescence is detected, it will most probably be due to spilled dye molecules in the extracellular matrix. Dyes that increase their fluorescence upon calcium binding will become brightly fluorescent in the interstitium. Therefore, great care should be taken not to spill dye while approaching the cell with the patch

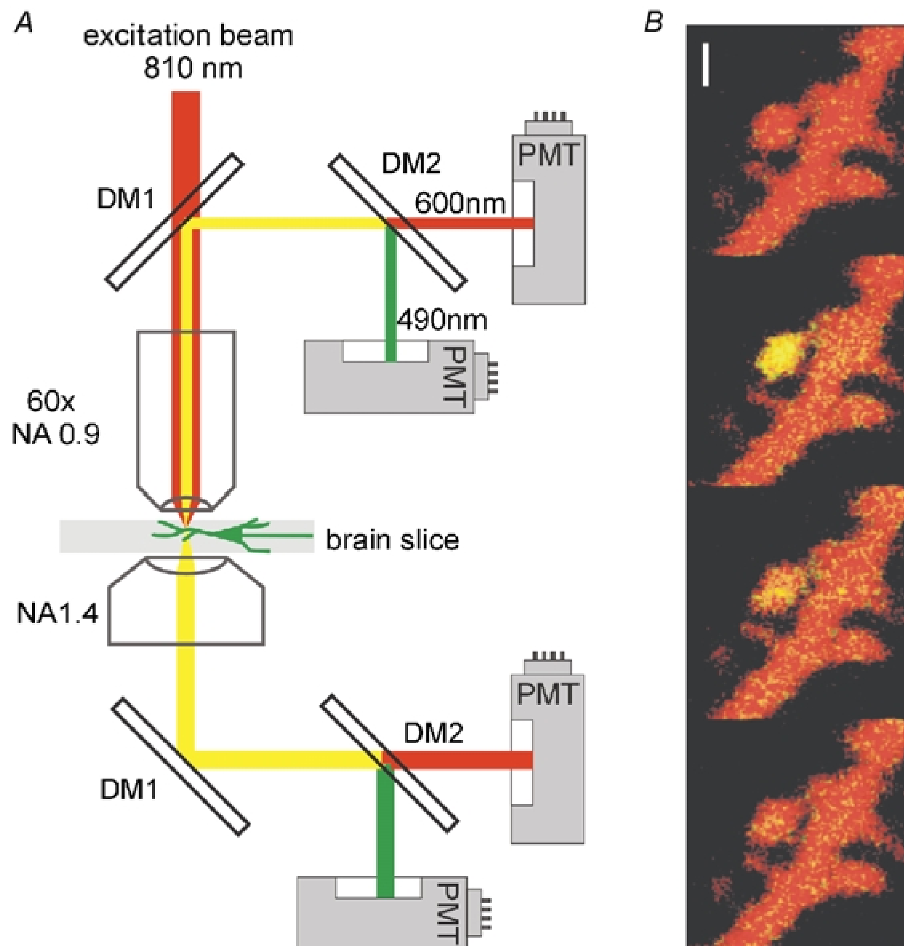

Figure 1

A, scheme of the fluorescence detection system used for slice physiology. An infrared laser beam (810 nm) is used to excite fluorescence in the preparation (scan mirrors not shown). Emitted photons are collected through the objective and through the condenser. Primary dichroic mirrors (DM1) separate the emitted photons from the excitation beam, secondary dichroic mirrors (DM2) are used to direct photons from the green and the red fluorescent dye to different photomultipliers (PMTs). Lenses and filters are omitted for clarity. *B*, calcium transient in a dendritic spine of a CA1 pyramidal cell in response to a single presynaptic action potential. Four frames (128 × 128 pixels) were acquired at a rate of 3.9 Hz (256 ms per frame). Presynaptic axons were stimulated after the first frame. Images are overlays of the anatomical image (Alexa 594, red) and the calcium-sensitive channel (Fluo5F, green; yellow in overlay). The signal from the green channel is displayed only within the dendrite using a binary mask generated from the anatomical image. Scale bar is 1 μm.

pipette (i.e. using low positive pressure and a quick approach by triangulation).

We measured postsynaptic calcium transients in dendritic spines to investigate NMDA receptor saturation and the statistics of glutamate release (Mainen *et al.* 1999*b*; Oertner *et al.* 2002). Pyramidal cells in the CA1 region of the hippocampus were filled with dye through a patch-clamp electrode and depolarized to relieve the voltage-dependent Mg^{2+} block of NMDA receptors, permitting NMDA receptor-mediated Ca^{2+} influx. Stimulation of several axons (Schaffer collaterals) with a monopolar glass electrode led to glutamate release from presynaptic terminals. The stimulus-induced postsynaptic calcium transients revealed the location of activated synapses on the dendrite (Fig. 1*B*). For maximal temporal resolution (2 ms), we used line scans across an active spine head and the parent dendrite. Since glutamate receptors are not saturated after the release of a single vesicle (Mainen *et al.* 1999*b*), different glutamate concentrations in the synaptic cleft lead to postsynaptic calcium transients of different amplitudes. We found that after successful synaptic transmissions postsynaptic calcium transients were larger under conditions of high release probability, indicating the frequent release of multiple vesicles in response to a single action potential (Oertner *et al.* 2002).

For quantitative calcium measurements we used the calcium-sensitive dye Fluo5F (Molecular Probes), which has a very large dynamic range (difference between calcium-bound and calcium-free fluorescence intensity). On the other hand, due to the very low resting calcium concentration in neurons, cells are almost non-fluorescent at rest, and fine anatomical details become invisible. Therefore, we added a red fluorescent dye to the intracellular solution (Alexa 594, not sensitive to calcium) to visualize better the anatomy of the cell. A secondary dichroic mirror and glass filters were used to separate the photons emitted from both dyes, which were detected in different photomultipliers (Fig. 1*A*).

This method of dual dye imaging has an additional advantage: to relate fluorescence changes to changes in intracellular calcium concentration, the measured change in fluorescence (ΔF) is usually normalized by the resting fluorescence ($\Delta F/F$; Grynkiewicz *et al.* 1985). Because of the low resting fluorescence of the Fluo series of dyes, even very low levels of background fluorescence (mainly due to calcium-bound dye molecules in the extracellular space) can introduce large errors in the calcium measurements. Subtraction of background fluorescence is not practical when imaging small structures like spines, because the background is non-homogeneous and the focal volume will encompass unknown fractions of extra- and intracellular space (for more detailed discussion see Sabatini *et al.* 2002). Instead of normalizing the calcium signal with respect to the resting fluorescence in the green channel, we divided the calcium signal (green channel) by the average intensity in the red channel (G/R ratio). The highly fluorescent red dye provides a robust intensity measurement for normalization of the calcium signal, making the G/R ratio independent of the spine volume. In addition, the G/R ratio can be readily calibrated in terms of absolute calcium concentrations. The calibration has to be done under the microscope, using electrodes with closed tips filled with the dye mixture at defined calcium concentrations.

The affinity and concentration of the calcium-sensitive dye has to be carefully chosen to match the expected amplitude of the calcium transients. In a neuron that has been filled with a calcium indicator, the amplitude of the free calcium transient ($\Delta[Ca^{2+}]_{free}$) is inversely proportional to the total buffering capacity, which is determined by the endogenous calcium buffering capacity of the cell (κ_e) and the calcium buffering capacity of the indicator (κ_{dye}):

$$\Delta[Ca^{2+}]_{free} = \frac{\Delta[Ca^{2+}]_{total}}{1 + \kappa_e + \kappa_{dye}},$$

where $\Delta[Ca^{2+}]_{total}$ is the total increase in calcium concentration (free and bound) after stimulation (Helmchen *et al.* 1996). In a typical imaging scenario, calcium buffering in the cell is completely dominated by the added calcium indicator ($\kappa_{dye} \approx 20\kappa_e$), so $\Delta[Ca^{2+}]_{free}$ is largely determined by the buffering capacity of the dye. As a rule of thumb, if $[Ca^{2+}] \ll K_D$, we can estimate κ_{dye} by dividing the concentration of the dye by its K_D for calcium: $\kappa_{dye} \approx [dye]/K_D$. (For the definition of κ, see Neher & Augustine, 1992.) In summary, under typical imaging conditions, doubling the concentration of the dye will reduce the peak amplitude of the free calcium transient by about 50 %.

For our measurements of NMDA receptor-mediated Ca^{2+} currents, we used a high concentration of a medium-affinity dye (600 μM Fluo5F; K_D, ~0.8 μM). Under these conditions, the calcium transient after synaptic stimulation with a single action potential did not saturate the dye. However, during a high frequency train of action potentials, calcium accumulated in the spine leading to dye saturation. The calcium dye competes with calcium extrusion mechanisms (Na^+–Ca^{2+} exchangers and Ca^{2+} pumps) and prolongs the calcium transients, leading to a summation of successive transients (Helmchen *et al.* 1996). The amplitude and time course of the calcium transient in the undisturbed cell can be predicted by extrapolation if the same experiment is repeated with dyes of different affinities, resulting in calcium transients of different amplitudes (Sabatini *et al.* 2002). When interpreting the spatial extent of the calcium signals, it must be kept in mind that calcium-bound dye molecules are free to diffuse in the cell, while endogenous dendritic calcium buffers are essentially immobile. Therefore, the lateral diffusion of the calcium-bound fluorescent dye is not equivalent to the lateral diffusion of free calcium ions in the absence of indicator dye.

Using two-photon microscopy, we could monitor the activity of single synapses in acute brain slices. The local

environment of a synapse can have a profound impact on its function. For example, glutamate released by presynaptic terminals is taken up by surrounding glial cells, reducing glutamate pooling and spillover between neighbouring synapses (Bergles *et al.* 1999). Communication between neurons and non-neuronal cells might be important for synaptic function and plasticity, demonstrated recently for the synapses between climbing fibres and Purkinje cells (Iino *et al.* 2001). Therefore, synapses between dissociated cells, while more readily accessible for imaging, will not necessarily display the same properties as synapses in intact brain tissue. For the study of synapses *in situ*, the optical sectioning capability of the two-photon microscope is a great asset.

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