

Dynamic Confocal Imaging of Living Brain

Dynamic confocal imaging in acute brain slices and organotypic slice cultures using a spectral confocal microscope with single photon excitation

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Confocal imaging in living brain slices allows the resolution of submicrometre structures of nerve cells, glia and brain vessels. Imaging living brain slices is in many respects different from conventional fixed histological preparations for which confocal microscopes were designed originally. Several problems (i.e. mechanical and thermal drift, and autofluorescence) resulting from the optical and structural properties of brain slices are discussed. Fluorescent indicators may be used to monitor numerous intracellular parameters such as pH and Ca^{2+} concentration, but not all of them are equally suitable for this type of work. Genetically engineered fluorescent proteins can be used to visualise the fine dendritic structure of neurones or track particular intracellular structures and proteins. They have also been used to generate indicators for Ca^{2+} , cAMP and other molecules. While conventional chemical indicators can be either loaded into neurones via patch pipettes or as membrane-permeable esters, protein indicators can be expressed in various types of cells using adenoviral vectors. Adenoviral transgenesis can be performed *in vitro* in both acute slices and organotypic slice cultures. Organotypic slice cultures give excellent optical access to neurones loaded with either conventional fluorescent indicators or transfected with adenovirus to express fluorescent proteins. They are most suitable for experiments where both conventional and genetically engineered indicators are combined. Single photon imaging in brain slices is limited to the superficial layers ($\sim 50 \mu\text{m}$), while multiphoton excitation has a much greater depth of penetration. However, the overall optical resolution achievable in single photon mode is at least as good as when using multiphoton excitation. *Experimental Physiology* (2002) **87.6**, 715–724.

Although initially used to resolve fine details in fluorescently labelled fixed histological preparations, confocal microscopes have more recently been applied to living tissues, such as brain tissue. This can be done in primary cultures grown on coverslips, in brain slices and even in brain *in vivo* (see for example other symposium papers in this issue; and Helmchen *et al.* 1999; Yoder, 2002). Several features of confocal scanning make it advantageous compared to conventional wide-field microscopy observations and measurements in brain slices. Probably the most important feature is that it allows the visualisation of much smaller objects (for example individual dendritic spines or axonal varicosities) embedded in the opaque brain tissue. Moreover, it also makes it possible to correctly interpret the 3-dimensional

organisation of fine brain structure, for example cell-to-cell contacts. Here we will discuss the technical aspects of confocal imaging in brain slices and some of the currently available fluorescent markers suitable for this type of work.

Application of confocal microscopy to living brain slices: problems and possible solutions

Minimising instability. The very idea of confocal imaging implies that the volume of material has to be scanned layer by layer. This process takes time: for a single plane scanned at reasonable resolution (512×512 pixels) a typical system nowadays requires somewhere between 0.3 and 0.6 s. Although it is possible to scan faster, one has to remember that faster scanning also means higher noise and lower sensitivity. Hence to obtain a high quality Z-stack of an

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object, such as a neurone (which may have a soma of $> 20 \mu\text{m}$ in the Z -direction, not to mention its processes), a considerable amount of time is needed, possibly up to several minutes.

The time required for imaging has important implications for work carried out in living brain slices as compared to the conventional histological preparations. Brain slices are soft and thick (usually $> 150 \mu\text{m}$) and during the period of observation some flattening may easily occur. This can lead to distortion in both the horizontal and vertical planes. Although hardly detectable with a CCD camera, a displacement of $\leq 1 \mu\text{m}$ in the Z -direction may completely distort data acquired using confocal imaging. Another side of the movement problem becomes evident when a fine structure must be observed for a long time, for example when monitoring Ca^{2+} dynamics or protein assembly in a dendritic spine. Because confocal microscopes by definition have a very narrow focal plane, keeping fine objects 'in focus' for many minutes or even hours is not easy in brain slices. To circumvent this a series of small Z -stacks can be taken and the appropriate images selected manually off-line.

In general we found that the thermal stability of the confocal microscope, its stage and the environment in which it is housed is an essential prerequisite for stable and high quality optical recordings. As a rule of thumb one can expect that a 1°C change in temperature of the rig will lead to a $1 \mu\text{m}$ displacement in one direction or another.

Moreover, thermal drifts tend to oscillate. To reduce thermal-related movements we clamped room temperature within $\pm 0.5^\circ\text{C}$.

An additional destabilising factor is the constant flow of artificial cerebrospinal fluid that is required to maintain brain slice viability. Moreover, during imaging the objective lens moves up and down slightly which can disturb the fluid level introducing further instability. It is also important to recognise that in order to minimise thermal drifts the objective lens needs to equilibrate with the bath solution in the recording chamber should there be a temperature difference. From our experience this takes a few minutes. Finally, the perfusion media are usually gassed with carbogen and it is essential to install in-line bubble traps after warming the media to the desired level. Bubbles can be a source of movement-related problems.

Scattering and absorbance of light in brain slice.

Compared to conventional histological preparations and cells grown on coverslips, brain slices are much more difficult to image due to their optical properties. The scattering and absorbance of light greatly depend on the age of the animals. For example, in the rat brainstem imaging becomes significantly more difficult after approximately 3 weeks of life due to the dramatic increase in myelination. The rapidly increasing optical density of the tissue is clearly more of a problem for single photon imaging and restricts the depth at which one can visualise cells without damaging tissue with excessive laser power.

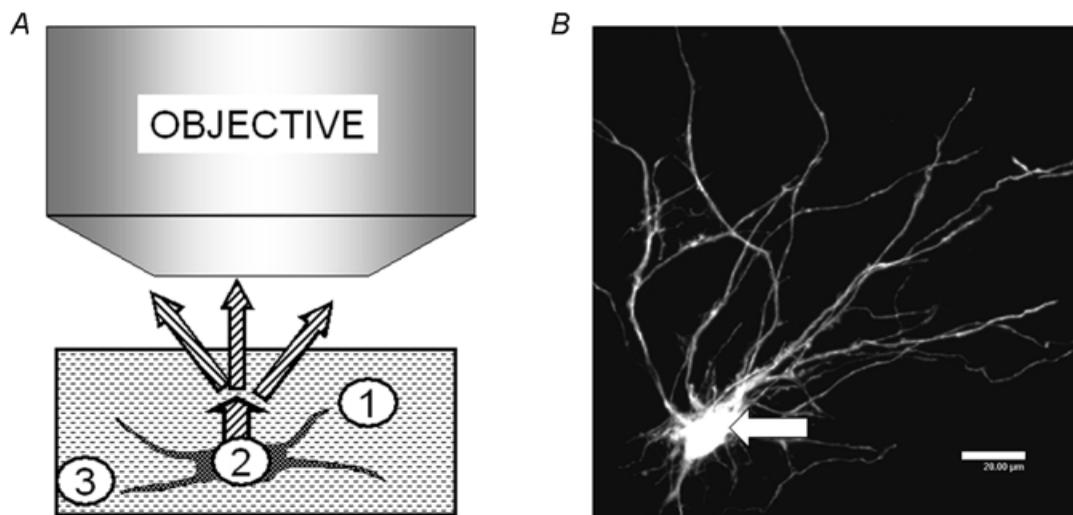


Figure 1

Specifics of imaging in the slice. *A*, light emitted by the fluorescent cell is absorbed and scattered by the tissue that surrounds the neurone. This results in loss of photons, which will be greater for the deeper parts of the cell ($3 > 2 > 1$). The effect of scattering (but not absorption) can be slightly reduced by using multiple detectors in multiphoton mode (see symposium paper by Tauer in this issue). In addition, different parts of the same neurone tend to have quite different concentrations of fluorescent indicators, such as eGFP. Absorption of light used for excitation on the way to the fluorescent object is greatly reduced in multiphoton systems. *B*, a maximum intensity projection of a $30 \mu\text{m}$ thick stack of images of an eGFP-expressing interneurone in a slice culture. 8-bit intensity encoding may be insufficient for imaging of living neurones: when imaging fine dendrites a bright area such as cell soma (arrow) may saturate (i.e. lack any detail). Scale bar represents $20 \mu\text{m}$.

Imaging a fluorescent neurone embedded in light absorbing tissue such as a brain slice may require a Z-stack over a depth of tens of micrometres to capture its dendrites. This causes a potential problem since fluorescence intensity varies strongly with depth such that top images will be much brighter than those obtained from within the tissue (Fig. 1A). Therefore, for quantitative measurements of fluorescence one has to take into account the effect of light absorbance. Light absorbance by tissue and the very different brightness of the visualised objects makes 8-bit intensity encoding present in many confocal microscopes a serious disadvantage. In our experience the 256 degrees of brightness (8 bits) are usually not enough to reveal every fine detail but avoid saturation from bright areas of the same neurone (Fig. 1B). In addition, both fluorescent chemical indicators and genetically encoded fluorescent proteins, introduced using viral vectors, are usually present at different concentrations in different cells and within different compartments (i.e. soma *vs.* dendrites) of the same cell. Attempts to compensate for the lack of dynamic range of an 8-bit system using mathematical compression algorithms (built into some software packages) in practice give no advantage. Hence, we believe that for confocal imaging in living brain slices a system with 12-bit intensity encoding with 4096 degrees of brightness is required.

Autofluorescence in brain slices. Even without a fluorescent dye brain slices contain fluorescent molecules. Some of these molecules, such as tyrosin, tryptophan and NADH will only be fluorescent when excited with UV light (< 400 nm). In single photon confocal imaging UV light is not normally used as it does not penetrate well into the tissue and damages living cells (note that the multiphoton excitation of these molecules is possible and this adds to the autofluorescence problem). The 488 nm light from an Ar laser (the most commonly used wavelength in a single photon system) excites a number of other endogenous molecules, in particular flavins and porphyrins which again can be excited in multiphoton systems. It is

important to note that most of these molecules emit in the green–yellow part of the spectrum (500–600 nm). This is unfortunate because the bulk of fluorophores used in confocal live slice imaging (most Ca²⁺ indicators and variants of enhanced green fluorescent protein (eGFP)) are imaged using 458 or 488 nm excitation and emit in the green part of the spectrum where both absorption and emission of endogenous molecules is the strongest (Fig. 2A; see König, 2001). As such the photomultipliers utilised in confocal microscopes are ‘colour-blinded’ and do not distinguish the colour in the same way as the human eye. Because of this, autofluorescence on the scanned images might be confused with a ‘true’ signal such as that emitted from eGFP expressed in a selected subset of neurones. However, a measure of the entire emission spectrum, as on a scanning spectral confocal microscope, will allow autofluorescence to be distinguished from a true eGFP signal (Fig. 2B). Alternatively, the ratio between emission at the GFP emission peak (500–540 nm) and above it (~550–600 nm), measured using appropriate filters, will give a predominant peak at 510–540 nm if eGFP is present.

Molecules that are mainly responsible for autofluorescence are mostly unexcitable by > 600 nm light (i.e. 633 nm line of red diode lasers). Thus, the red part of the spectrum seems to be ideal for single photon imaging of difficult and deep objects in brain slices where autofluorescence is intense. Unfortunately, at present there are not many far-red-shifted fluorophores which could be applied to living slices, an exception being DiD, a lipophilic tracer from Molecular Probes. Obviously, using longer wavelengths will slightly reduce the achievable limits of optical resolution.

Combining confocal and transmitted light imaging. Differential interference contrast (DIC) is commonly used nowadays for visualised patch clamp recordings from single neurones in brain slices. Conventionally infra-red light is used in order to minimise scattering and obtain

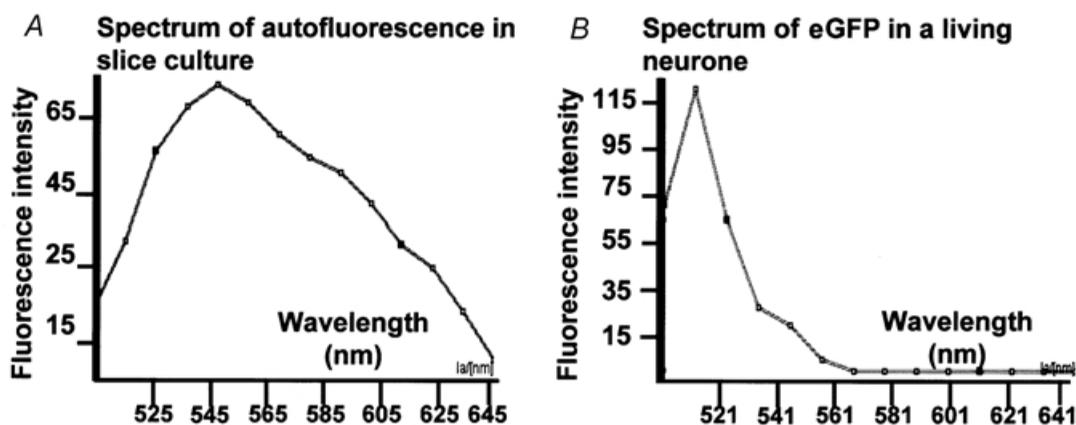


Figure 2

Autofluorescence and spectrum of eGFP in living neurones. A, spectrum of autofluorescence in a slice culture using 488 nm excitation. B, spectrum of eGFP expressed in a neurone using adenoviral gene transfer (X-axes in A and B have slightly different scales for technical reasons).

crisp images. The polarised beam from the condenser passes through the slice and is collected by the objective. Many confocal microscopes are equipped with an inverted version of 'DIC-like' optics. The same laser beam as used to excite a fluorophore is sampled by an additional device (a photodiode or photomultiplier) placed underneath the condenser, which in this case performs the function of an objective. As a result one can obtain a scanned image of non-fluorescent objects. Although we have tried different laser lines the best penetration in an opaque tissue such as a slice was obtained using the red diode (633 nm line).

The DIC-like optics is a useful feature for various reasons. It may help to visualise structures and cells, which are difficult to load with fluorescent indicators. In our experiments we needed to image endothelial cells in numerous capillaries embedded in brainstem slices. Although the slices were loaded with Ca^{2+} indicators it proved extremely difficult to use confocal fluorescent images alone to identify these small structures ($\leq 10 \mu\text{m}$ in the rat) within the tissue. However, using the DIC optic we frequently found erythrocytes trapped in blood vessels (Fig. 3). Thus, overlaying the DIC and the confocal fluorescent images on-line greatly facilitated their interpretation. Additionally, transmitted light detectors can be used in patch clamp experiments to visualise the surface of the slice and the tip of patch pipettes prior to seal formation.

Fluorescent indicators for confocal imaging in living brain slices

Fluorescence occurs when a molecule absorbs one or more photons to reach the excited state from which it can relax to the ground state; this process is accompanied by emission of a photon. Confocal imaging in brain slices requires that the cells or their elements must be fluorescent. We will briefly discuss the ways by which this can be achieved and some of the complications arising from the use of the brain slices.

Conventional indicators for confocal imaging. Which fluorophores can be used for imaging of living brain? First, molecules can change their fluorescent properties in response to some intracellular parameter such as Ca^{2+} concentration. These are known as fluorescent indicators. The most popular fluorescent dyes are used to measure intracellular Ca^{2+} concentration; these include dyes such as Ca Green, Oregon Green, Ca Orange, Fluo 3, 4 and 5. All of these dyes have one general feature: they can be effectively excited by the blue 488 nm Ar laser line and their fluorescence increases upon Ca^{2+} binding. (Note there are exceptions where dyes show a decrease in the fluorescence.) Ca^{2+} indicators have different affinities for Ca^{2+} and their kinetics are also very different. Low affinity 'fast' dyes of the Fluo family are usually used for fast events such as action potential-evoked Ca^{2+} transients. Dyes with higher affinity such as Ca Green or Oregon Green will saturate if Ca^{2+} concentration approaches the micromolar range near sites of Ca^{2+} release or Ca^{2+} entry.

Another important parameter that varies between indicators is the dynamic range, that is the maximal change in fluorescence between the free and Ca^{2+} -bound state (Fig. 4A). In theory it seems obvious that the higher dynamic range automatically means a better indicator. However, in reality this usually means that dyes with the highest dynamic range will be dim or even invisible unless they are Ca^{2+} bound. As a result it may be very difficult to see cells especially those embedded deep within the tissue unless they are stimulated to trigger Ca^{2+} release (Fig. 4B). This may be restrictive especially if multiple challenges to provoke Ca^{2+} elevations need to be avoided. An elegant way around this problem is to load cells via a patch pipette with a mixture of a green Ca^{2+} indicator (e.g. Fluo-4 or Fluo-5) and a bright red-shifted non-toxic neutral dye, such as one of the red members of the Alexa family (Sabatini *et al.* 2002). These dyes have well-separated spectra and can be sampled with minimal overlap. The red

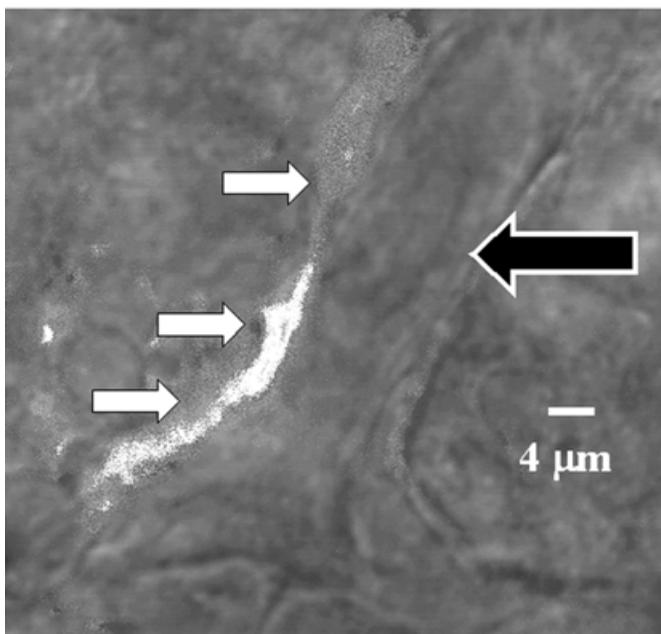


Figure 3

Combination of transmitted light differential interference contrast (DIC) and confocal fluorescence imaging helps to reveal microvasculature in an acute brain slice. A brainstem slice was loaded using Fluo-4 AM. A bifurcating capillary with a trapped concave erythrocyte (black arrow) can be seen (DIC image) superimposed on the fluorescent signal from the endothelial cells in the left wall of the capillary (white arrows).

channel therefore can be used to focus on small objects (such as dendritic spines), which in their resting state have relatively low Ca^{2+} levels and therefore low Fluo-4 fluorescence.

Fluorescent dyes all suffer from the same disadvantage: they bleach under intense illumination. There is a popular misconception that multiphoton imaging is less prone to bleaching. This is not correct and in fact for some dyes bleaching occurs even faster under multiphoton excitation (see symposium paper by Tauer in this issue; and Dickinson & Fraser, 2001). The difference between these two modes is in *where* the bleaching occurs. In single photon mode maximal excitation (and bleaching) occurs not only in the focal plane but also in the tissue above and below it. Multiphoton excitation is only possible within the focal plane, which bleaches fast, but there is virtually no bleaching above or below this point. If the dye is loaded into the neurone from a patch pipette it can be replenished by diffusion, but if it has been loaded extracellularly in the membrane-permeable acetoxymethyl ester (AM) form, bleaching may become a problem, especially when imaging very small objects.

Bleaching introduces a drift into the baseline fluorescence signal but can, to a degree, be counteracted using ratiometric dyes. Such dyes respond to a change in a physiological variable by a change in their light absorption or light emission characteristics. The first scenario applies to the popular Fura-2 broadly used in conventional Ca^{2+} measurements. Unfortunately, as yet there is no comparable ratiometric Ca^{2+} dye suitable for confocal microscopy: for ratiometric measurements using Fura-2, two alternating UV wavelengths are required for excitation which the currently available confocal microscopes cannot provide. A solution to this problem would be to have dyes that would respond to a change in the measured variable by a shift in their emission spectra. At present these are not available for Ca^{2+} but there is a pH-sensitive indicator, SNARF that gives a good example of a ratiometric dye suitable for confocal imaging. SNARF can be excited by 488 nm light and its emission peak depends on the pH (Fig. 4C). Therefore one can simultaneously image at two wavelengths (one at the frequency where the emission is maximal at low pH, and the other at the high pH) and take the ratio as an index of pH (see Fig. 4D). Clearly, bleaching

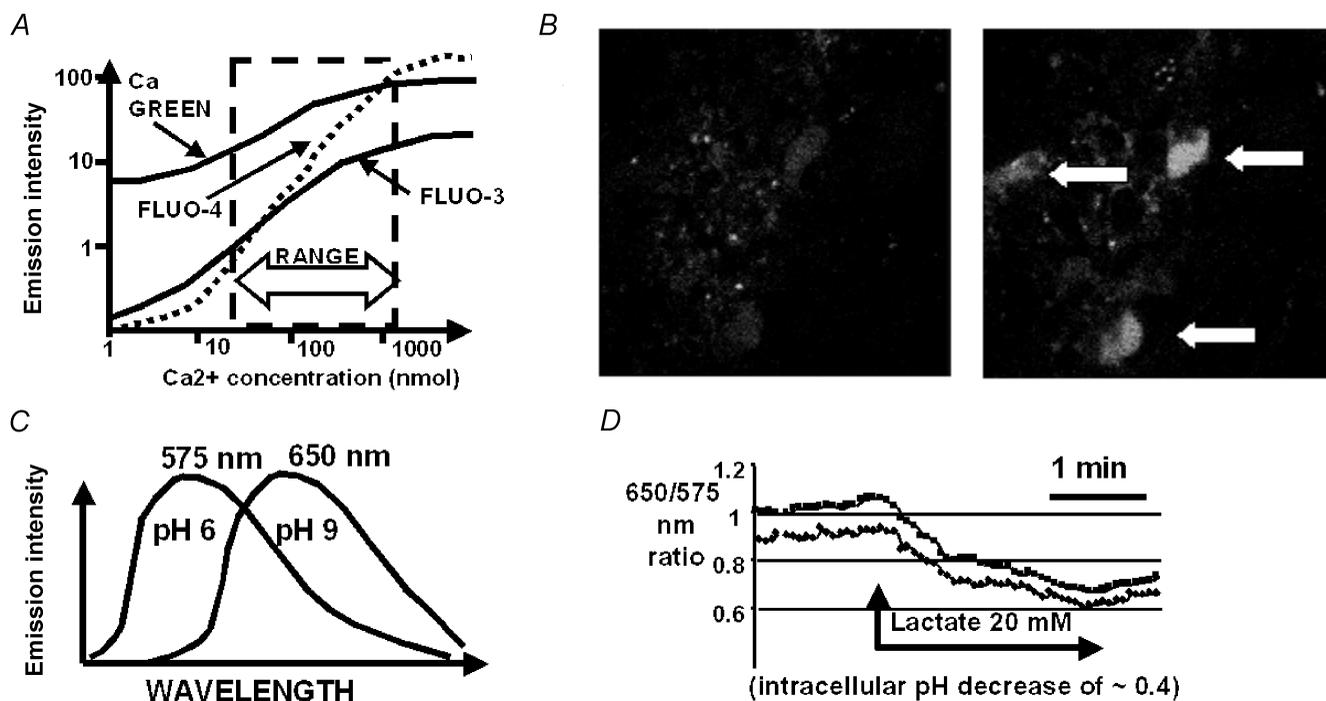


Figure 4

Fluorescent indicators with different dynamic ranges and saturation characteristics. *A*, the Ca^{2+} concentration changes the emission of three Ca^{2+} indicators within the physiological range of Ca^{2+} concentrations (double-sided arrow) in a particular cell. Because neurones have relatively low free Ca^{2+} concentrations, indicators with a large dynamic range such as Fluo-4 may be too dim to be imaged when cells are at rest. *B*, imaging of the intracellular Ca^{2+} concentration using Fluo-4-AM: cells might be too dim in their resting state. In this case a 20 s pulse of 30 mM KCl to trigger depolarisation and Ca^{2+} influx reveals neurones which were invisible before (arrows). *C*, confocal ratiometric measurements of intracellular pH using SNARF-1-AM are possible because the emission spectrum of this dye changes in response to pH. *D*, response to bath application of lactate in two individual brainstem neurones measured using SNARF-1. Excitation was achieved with the 488 nm line and emission was sampled at 650 and 575 nm.

in this case will have little effect because unidirectional changes in both channels will cancel each other out.

Genetically engineered indicators for confocal imaging.

An ideal indicator should be ratiometric, bright, photostable, it should be easy to load into any cell type and report any intracellular variable we might be interested in. It is surprising that in spite of the vast number of known chemicals there are only very few really good indicators. Therefore the announcement of the genetically engineered indicators based on mutants of eGFP evoked huge interest within the scientific community. In 1997 Persechini *et al.* and Miyawaki *et al.* published pioneering reports describing Ca^{2+} indicators utilising fluorescence resonance energy transfer (FRET) between the cyan and the yellow mutants of eGFP (CFP and YFP, respectively). In both cases the molecules included a Ca^{2+} sensor that changes conformation upon Ca^{2+} binding due to an interaction of the Ca^{2+} -binding domain of calmodulin and a fragment of the myosin light chain kinase. This Ca^{2+} -sensing element was engineered to change the distance between CFP and YFP thereby transferring blue photons from CFP to YFP, resulting in FRET (i.e. a decrease in CFP emission and an increase in YFP emission). The idea has tremendous potential because the principle as such can be applied to nearly any other intracellular messenger or metabolite such as nitric oxide (Pearce *et al.* 2000) or cAMP (Zaccolo *et al.* 2000). Moreover, problems regarding adequate loading of fine processes and bleaching are reduced since the dye is made intrinsically.

During the last few years several groups including ours have tried to use these fusion proteins for confocal imaging in living slices using adenoviral vectors for gene delivery. Animals were either pre-transfected a few days before the experiment or acute slices were transfected by incubation with adenoviral vectors (C. Stokes & S. Kasparov, unpublished data). Several problems were encountered

that complicated the application of these indicators and made obtaining reliable data very difficult. One problem was that most currently available confocal systems are not equipped with lasers capable of generating light close to 430 nm. This wavelength is thought to give the best separation of CFP/YFP FRET pairs because it excites YFP much less than CFP. We therefore had to use the 458 nm line from the Ar laser. In the original publications where 430 nm excitation wavelength was used these constructs had a dynamic range of only ~20%. Figure 5 shows the spectra of YC2.1 (Miyawaki *et al.* 1997) in three cells with different levels of YC2.1 expression, obtained using the spectral feature of the Leica SP system. It can be seen that in all cases YFP is much brighter than CFP but in living neurones their spectra overlapped instead of forming two separate peaks. Thus, it is possible that the use of suboptimal excitation resulted in responses which in some cases were too small and failed to be significant. A second issue is that the expression of $> 10 \mu\text{M}$ eGFP in neurones may result in cell damage and death (S. Kasparov & C. Stokes, unpublished observation). Thus, a possibility is that differentiated neurones within brain slices did not allow an optimal FRET response because high concentrations of eGFP mutants are damaging. (It needs to be emphasised that neurones expressing low micromolar concentrations of eGFP can survive *in vivo* for many months.) Thirdly, as discussed recently the intrinsic ability of the eGFP mutants used in these constructs to form oligomers could interfere with FRET; this might be overcome with the new generation of 'monomeric' eGFPs (Zacharias *et al.* 2002). Another interesting development is the introduction of the 'split' mutants of eGFP and CFP which either acquire or lose fluorescence upon binding with Ca^{2+} (Nakai *et al.* 2001). These indicators do not require exotic excitation wavelengths and might have a better dynamic range than the currently available FRET constructs. Finally, it is worth mentioning that the recent versions of the red fluorescent

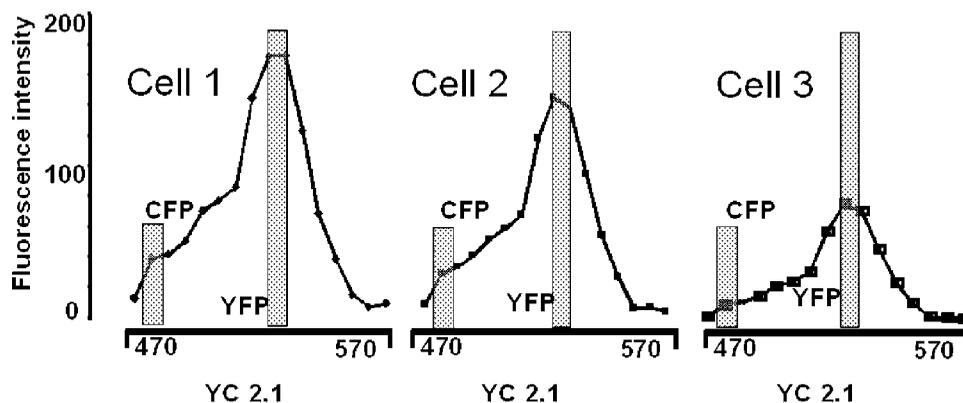


Figure 5

Spectra of a genetically engineered fluorescence resonance energy transfer (FRET) Ca^{2+} indicator YC2.1 in three individual cells measured using 458 nm Ar laser line for excitation. Note that the spectra of the CFP and YFP present in this construct strongly overlap under these conditions. Grey boxes indicate theoretical emission peaks of CFP and YFP incorporated into the molecule of YC2.1.

protein dsRed have improved characteristics and apparently this family of fluorescent indicators may be of great value for multicolour *in vivo* imaging (Gurskaya *et al.* 2001; Yanushevich *et al.* 2002).

Delivery of fluorescent marker into neurones in brain slice

Fluorescent chemical dyes can be loaded into neurones through patch pipettes or using membrane-permeable AM forms of these dyes that enter through the lipid membranes and become trapped within the cell. Both methods have been described in numerous publications and reviewed elsewhere (for example, Takahashi *et al.* 1999). Compared to conventional imaging with a CCD camera, confocal imaging in AM-loaded cells within a brain slice gives some advantage. The surface of an acutely cut slice is always covered with cellular debris, which contains a lot of free Ca²⁺ and is therefore always fluorescent. However, using confocal optics the noise from this superficial layer can be excluded. In this way we were able to measure Ca²⁺ transients in cells embedded within a brainstem slice (Paton *et al.* 2001a). Figure 3 shows an individual endothelial cell of a capillary that was imaged after loading with Fluo-4 in the nucleus of the solitary tract of an acute slice.

Genetically engineered fluorescent proteins (e.g. eGFP) may be expressed chronically and acutely in many cell types contained in living brain and brain slices, respectively. Recently, we have used adenoviral vectors for gene delivery for *in vivo* and *in vitro* experiments (Paton *et al.* 2001b; Wong *et al.* 2002). For imaging of cells from acute slices adenoviruses need to be injected into the relevant part of the brain > 3 days prior to making a slice preparation. This is relatively straightforward in mature animals but may be impossible in very young pups. Therefore the transfection in advance strategy has its limitations because brain tissue becomes increasingly much less transparent with age. This restricts the maximal depth at which cells of interest can be imaged thereby reducing the number of fluorescent cells within the reach of the optics. (Multiphoton imaging is advantageous here due to the better penetration of the infrared light used and other technical features that minimise both the loss of light and scattering. See symposium papers by Tauer; Holthoff & Tsay; and Helmchen in this issue.) In practice finding a suitable transfected cell is a matter of chance and if the density of these cells is high enough, even in slices of mature animals it is possible to get high quality images of objects < 1 µm in cross-section (i.e. fine dendrites of neurones or an endothelial cell; Fig. 6A and B).

Since *in vivo* gene transfer becomes increasingly more difficult in young rat pups, in which the brain is optically optimal, we have developed alternative strategies. First, adenoviral vector transfection of acute slices can be performed immediately after slice cutting. Application of adenoviral vectors with constructs under the control of an active promoter, such as human cytomegalovirus

promoter (HCMV) causes accumulation of micromolar concentrations of eGFP in transfected neurones and glia within hours – when the slice is still fully viable. This method will be described in detail in a separate communication. However, such ultra-rapid expression requires the most active promoters, e.g. HCMV, which are not selective for any particular type of neurone. If a fluorescent transgene has to be targeted to a particular neuronal phenotype with a cell-specific promoter that is far less active, then the lifetime of the acute slice may be insufficient for transgene expression. In this scenario, organotypic slice cultures may be indispensable.

Organotypic slice culture

Slices prepared from essentially any area of brain of young (typically 7- to 8-day-old) pups can be maintained on suspended 0.4 µm pore membranes at the interface between culture media and 5% CO₂ atmosphere for several weeks. During that time they flatten and transform into organotypic cultures. However, they maintain nuclear boundaries and brain ventricles and in this regard are similar to acute brain slices. For example, the characteristic laminar layout of the hippocampus can be recognised. Moreover, characteristic neuronal phenotypes (e.g. pyramidal cells in hippocampus) can be easily identified after weeks in slice culture indicating that cells in this preparation de-differentiate much less than in primary cultures. (We acknowledge that re-organisation does occur in slice cultures.)

Organotypic slice cultures offer excellent conditions for both imaging and adenoviral transgenesis. Although they are not much more transparent than brain tissue of a young animal, they are much thinner than any conventional acutely cut slice. The thickness of a slice in culture depends on the thickness of the original slice and the time *in vitro* and eventually only a few cell layers remain. Altogether, slice cultures are more mechanically stable and less prone to drift compared with acute slices that show flattening within the time course of collecting images. Hence, it is easier to keep small objects in focus and take accurate Z-stacks with submicrometre steps. Additionally, cultured slices have flat and relatively 'clean' surfaces that reduce light scattering compared with acute slices where the surface is always covered with cellular debris. For these reasons, cultured slices often give a better clarity of image.

Slice cultures can also be efficiently transfected with adenoviral vectors. In this respect our results contrast somewhat with the recent communication by Ehrenguber *et al.* (2001). We have successfully transfected slice cultures from various brain areas (hippocampus, neocortex, hypothalamus and brainstem) with adenoviruses using four different promoters, including HCMV. Numerous neurones with extensive dendritic trees and widespread axons could be visualised in great detail (Fig. 1B). Moreover, in slice cultures it is also possible to combine the virally mediated gene expression with application of vital dyes that reveal details of intercellular connectivity (Fig. 6C and D). A full

account of these methods and data is out of scope of this communication and will be presented elsewhere.

Limitations of confocal imaging: how deep and how small?

In our experience conventional single photon confocal imaging of cells within acute brain slices is limited to the upper 60 μm . In fact most of our high quality images were obtained at depths $\leq 40 \mu\text{m}$. This reflects, in part, light scattering and opaqueness of the tissue as described above. Notwithstanding, it is possible to see fluorescent somata of neurones and glia at 100 μm below the surface but light scattering prevents visualisation of distal dendrites and spines. From published data small dendrites and even dendritic spines ($\sim 1\text{--}5 \mu\text{m}$) can be imaged at depths $\geq 100\text{--}200 \mu\text{m}$ using multiphoton microscopes. Multiphoton imaging has been successfully used in acute and cultured slices (Dunaevsky *et al.* 1999; Engert & Bonhoeffer,

1999; Cox *et al.* 2000; Sabatini *et al.* 2002). The real potential of the multiphoton approach is demonstrated by the application of this method *in vivo* (Helmchen *et al.* 1999, also this issue). At the same time, for the reasons explained above, the depth of imaging appears not to be a major limiting factor for single photon imaging in our acute or cultured slice preparations.

Compared with multiphoton imaging, the single photon system appears to be at least as powerful if not better in terms of imaging small structures with excellent clarity. The smallest clearly visible structures visualised using multiphoton systems are of dendritic spines of neurones filled via a patch pipette (Sabatini *et al.* 2002; see also other contributions in this volume). The latter allows maximal contrast between the fluorescent cell and the background. The smallest structures in these images are typically in the range 0.7–0.8 μm . As shown in Fig. 6C and D single

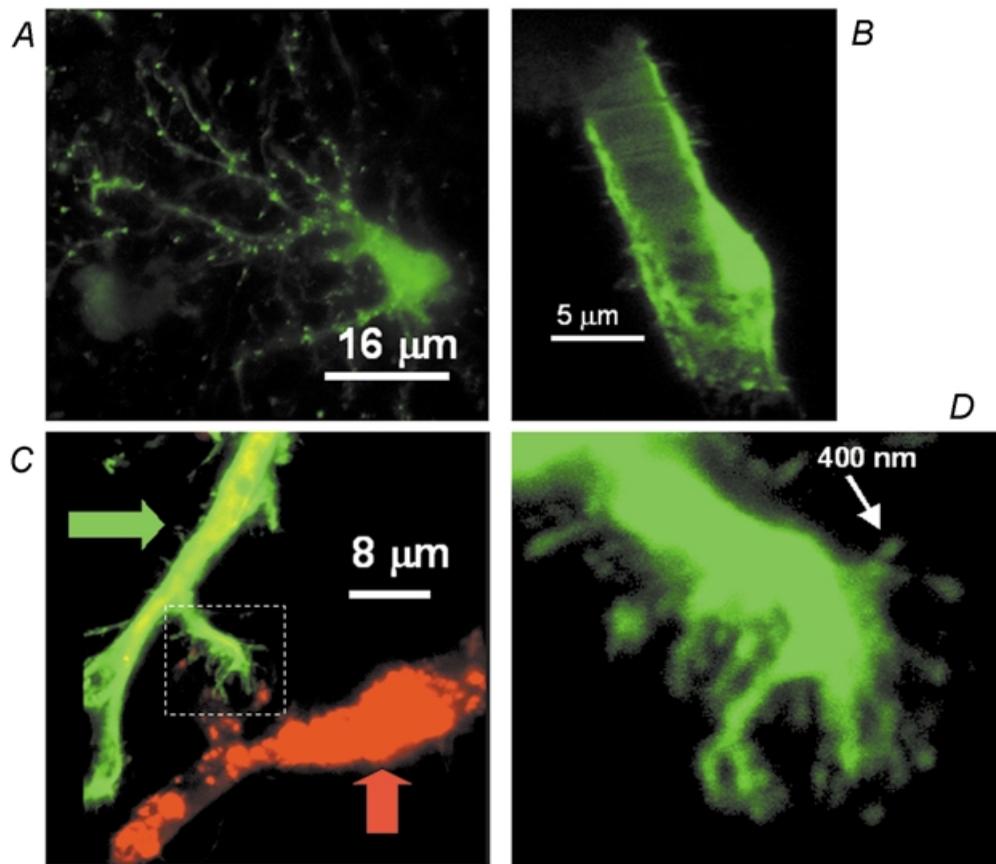


Figure 6

Optical resolution achievable using single photon confocal imaging in acute slices and organotypic slice culture. *A*, an eGFP-expressing neurone in nucleus of the solitary tract (NTS) visualised in a pre-transfected acutely cut brain slice. *B*, an endothelial cell expressing eGFP in cross-section visualised in a pre-transfected acutely cut brain slice. *C*, in hippocampal slice culture a pyramidal cell was visualised using Dil (red arrow) and an interneurone was targeted using an adenoviral vector to express eGFP (green arrow). This maximum intensity projection stack shows an area of contact between the processes (dendrites?) of these two cells. *D*, high power image of the contact between the two cells shown in *C*. eGFP channel only; note that the smallest visible details (arrow) are only $\sim 400 \text{ nm}$ in cross-section.

photon imaging of eGFP-expressing neurones permits visualisation of structures in the range of 0.4 μm (at least in X–Y dimensions) with the density of 256 \times 256 or even 512 \times 512 pixels. Moreover, resolution in the Z-direction is sufficient to obtain high density XYZ-stacks sufficient for high quality 3-dimensional reconstructions and animated rotations. This is consistent with the view that multiphoton confocal microscopy does not enhance optical resolution, especially in the Z-direction (for discussion, see symposium paper by Tauer in this issue; and also Dickinson & Fraser, 2001; Wallace *et al.* 2001). In addition, we obtain most of our images using < 2–3 mW power from the 488 nm laser line. This compares with the ≥ 10 mW commonly required to produce the two-photon effect. Altogether, it seems that for imaging in acute slices, and especially in cultured slices, a high quality single photon system is more than adequate.

Conclusions

Confocal imaging from either acute brain slices or organotypic slice cultures offers an analysis of the structure and function of differentiated neurones with submicrometre resolution. The problems of mechanical instability and the less than optimal optical properties of slices do present numerous technical challenges before high-resolution images can be obtained. However, both chemical and genetically engineered fluorophores can be used for the dynamic imaging of intracellular processes within living neurones. The hope for the future is that even better fluorescent indicators will appear, for example, a red-shifted ratiometric Ca^{2+} indicator suitable for confocal imaging would be of great value. At present, chemical fluorescent indicators appear to be more reliable than calcium-sensitive FRET fusion proteins. However, constructs based on fluorescent proteins have enormous potential. Finally, organotypic slice cultures offer excellent conditions for imaging and also for gene delivery using adenoviral vectors.

- COX, C. L., DENK, W., TANK, D. W. & SVOBODA, K. (2000). Action potentials reliably invade axonal arbors of rat neocortical neurons. *Proceedings of the National Academy of Sciences of the USA* **97**, 9724–9728.
- DICKINSON, M. E. & FRASER, S. E. (2001). Multiphoton laser scanning microscopy and dynamic imaging in embryos. In *Methods in Cellular Imaging*, ed. PERIASAMY, A., pp. 200–215. University Press, Oxford.
- DUNAIEVSKY, A., TASHIRO, A., MAJEWSKA, A., MASON, C. & YUSTE, R. (1999). Developmental regulation of spine motility in the mammalian central nervous system. *Proceedings of the National Academy of Sciences of the USA* **96**, 13438–13443.
- EHRENGRUBER, M. U., HENNOU, S., BUELER, H., NAIM, H. Y., DEGLON, N. & LUNDSTROM, K. (2001). Gene transfer into neurons from hippocampal slices: comparison of recombinant Semliki Forest Virus, adenovirus, adeno-associated virus, lentivirus, and measles virus. *Molecular and Cellular Neuroscience* **17**, 855–871.
- ENGERT, F. & BONHOEFFER, T. (1999). Dendritic spine changes associated with hippocampal long-term synaptic plasticity. *Nature* **399**, 66–70.
- GURSKAYA, N. G., FRADKOV, A. F., TERSKIKH, A., MATZ, M. V., LABAS, Y. A., MARTYNOV, V. I., YANUSHEVICH, Y. G., LUKYANOV, K. A. & LUKYANOV, S. A. (2001). GFP-like chromoproteins as a source of far-red fluorescent proteins. *FEBS Letters* **507**, 16–20.
- HELMCHEN, F., SVOBODA, K., DENK, W. & TANK, D. W. (1999). *In vivo* dendritic calcium dynamics in deep-layer cortical pyramidal neurons. *Nature Neuroscience* **2**, 989–996.
- KONIG, K. (2001). Cellular response to laser radiation in fluorescent microscopes. In *Methods in Cellular Imaging*, ed. PERIASAMY, A., pp. 236–256. University Press, Oxford.
- MIIYAWAKI, A., LLOPIS, J., HELM, R., MCCAFFERY, J. M., ADAMS, J. A., IKURA, M. & TSIEN, R. Y. (1997). Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature* **388**, 882–887.
- NAKAI, J., OHKURA, M. & IMOTO, K. (2001). A high signal-to-noise Ca^{2+} probe composed of a single green fluorescent protein. *Nature Biotechnology* **19**, 137–141.
- PATON, J. F. R., BOSCAN, P., MURPHY, D. & KASPAROV, S. (2001a). Unravelling mechanisms of action of angiotensin II on cardiorespiratory function using *in vivo* gene transfer. *Acta Physiologica Scandinavica* **173**, 127–137.
- PATON, J. F. R., DEUCHARS, J., AHMAD, Z., WONG, L.-F., MURPHY, D. & KASPAROV, S. (2001b). Adenoviral vector demonstrates that angiotensin II-induced depression of the cardiac baroreflex is mediated by endothelial nitric oxide synthase in the nucleus tractus solitarius of the rat. *Journal of Physiology* **531**, 445–458.
- PEARCE, L. L., GANDLEY, R. E., HAN, W., WASSERLOOS, K., STITT, M., KANAI, A. J., MCLAUGHLIN, M. K., PITTS, B. R. & LEVITAN, E. S. (2000). Role of metallothionein in nitric oxide signaling as revealed by a green fluorescent fusion protein. *Proceedings of the National Academy of Sciences of the USA* **97**, 477–482.
- PERSECHINI, A., LYNCH, J. A. & ROMOSER, V. A. (1997). Novel fluorescent indicator proteins for monitoring free intracellular Ca^{2+} . *Cell Calcium* **22**, 209–216.
- SABATINI, B. L., OERTNER, T. G. & SVOBODA, K. (2002). The life cycle of Ca^{2+} ions in dendritic spines. *Neuron* **33**, 439–452.
- TAKAHASHI, A., CAMACHO, P., LECHLEITER, J. D. & HERMAN, B. (1999). Measurement of intracellular calcium. *Physiological Reviews* **79**, 1089–1125.
- WALLACE, V. P., DUNN, A. K., COLENO, M. H. & TROMBERG, B. J. (2001). Two-photon microscopy in highly scattering tissue. In *Methods in Cellular Imaging*, ed. PERIASAMY, A., pp. 180–199. University Press, Oxford.
- WONG, L.-F., POLSON, J. W., MURPHY, D., PATON, J. F. & KASPAROV, S. (2002). Genetic and pharmacological dissection of pathways involved in the angiotensin II-mediated depression of baroreflex function. *FASEB Journal* **16**, 1595–1601.
- YANUSHEVICH, Y. G., STAROVEROV, D. B., SAVITSKY, A. P., FRADKOV, A. F., GURSKAYA, N. G., BULINA, M. E., LUKYANOV, K. A. & LUKYANOV, S. A. (2002). A strategy for the generation of non-aggregating mutants of Anthozoa fluorescent proteins. *FEBS Letters* **511**, 11–14.
- YODER, E. J. (2002). *In vivo* microscopy of the mouse brain using multiphoton laser scanning techniques. In *Multiphoton Microscopy in The Biomedical Sciences*, vol. II, ed. PERIASAMY, A. & SO, P. T. C., pp. 14–29. The Society of Photo-Optical Instrumentation Engineers in Bellingham, Washington, USA.

- ZACCOLO, M., DE GIORGI, F., CHO, C. Y., FENG, L., KNAPP, T., NEGULESCU, P. A., TAYLOR, S. S., TSIEN, R. Y. & POZZAN, T. (2000). A genetically encoded, fluorescent indicator for cyclic AMP in living cells. *Nature Cell Biology* **2**, 25–29.
- ZACHARIAS, D. A., VIOLIN, J. D., NEWTON, A. C. & TSIEN, R. Y. (2002). Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science* **296**, 913–916.

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