

ADVANCED TRACER TECHNIQUES TO MONITOR SYNAPTIC ACTIVITY

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INTRODUCTION

Up to a few years ago the only way to look at exo-endocytosis in neuronal cells was to record from the cell body of the postsynaptic element the electrical response produced by synaptic release of neurotransmitter molecules. On a large scale, these types of responses have been fundamental in helping us to understand the brain circuitry. On a more microscopical scale, since these responses typically reflect the activity of many up to several hundreds synaptic contacts, they are not sufficiently informative when precise information about the exo-endocytotic process at small terminals is needed. In the last 10-15 years many novel methodologies have become available to study more directly exo-endocytosis at individual synapses. These include fluorescent dyes (2, 4-6, 16-18, 21, 22, 25), antibodies (7, 11-13), genetically encoded probes (14, 15, 20). Here we will review the two most popular labelling techniques, FM dyes and antibodies directed against intraluminal epitopes of synaptic vesicle proteins. We will review their use including optical analysis, and address some important caveats we need to be aware of.

FM DYES

FM1-43 (21) is an amphiphilic styrylpyridinium molecule with a lipophilic tail (two aliphatic hydrocarbon chains attached to an aminophenyl group) and a positively charged head (a pyridinium group). The fluorophore group is composed of two aromatic rings linked together by a double bond bridge. The excitation and emission peaks are located respectively at 500 nm and 625 nm (21). The lipophilic tail allows FM1-43 to partition in the plasma membrane but the molecule cannot cross the lipid bilayer of the synaptic vesicle due to the large cationic head. Once incorporated in the membrane, the interaction with lipid molecules causes a large increase in brightness (~ 30-fold increase in quantum efficiency) with a strong fluorescent staining of the surface of all exposed cells. As mentioned above, during release of neurotransmitter through exocytosis, synaptic vesicles briefly expose their internal face to the outside world. If FM1-43 is present in the external environment, the exposed vesicle membranes will be stai-

ned and when recycled back, FM1-43 will get trapped inside the synaptic vesicles and the terminal stained. Unfortunately the synaptic terminal fluorescence becomes visible only after an extensive cell wash-out with physiological saline solution to remove the aspecific staining from FM1-43 incorporated in external membranes. This washing phase usually lasts for many minutes and it must be assumed that no FM1-43 is lost from synaptic vesicles during this phase. In some experiments, mainly to visualize FM1-43 staining in brain slices, destaining has been improved by extracellular quenching agents. During the loading phase of FM1-43, dye entry through synaptic vesicles will greatly exceed the constitutive bulk flow uptake via endosomes only when Ca^{2+} regulated exocytosis is augmented for a brief period of time and many different stimulation protocols have been devised, including repetitive electrical stimulations of presynaptic afferents (2, 5, 17) and more elementary stimulation maneuvers. Under these circumstances, FM1-43 accumulates inside synaptic vesicles as demonstrated by EM analysis following dye photoconversion. FM1-43 has been successfully tested in a very large number of different types of nerve terminals including the neuromuscular junction (4-6), hippocampal (2, 16-18, 22), and types of neurons. In some of these experiments, FM1-43 has been simply utilized as a tool to visualize and to map the location of living synaptic boutons, something unimaginable just a few years ago. But, most importantly, FM1-43 has been extensively used for the investigation of kinetics of exo-endocytosis at the level of individual synaptic terminals. This dye has produced significant advances in our understanding of many aspects of synaptic transmission, especially at the level of the neuromuscular junction and of cultured hippocampal synapses. Some important aspects of this technique must be underlined here to better understand the use of this dye at central synapses. During intense stimulation protocols, the amount of FM1-43 taken up by individual hippocampal terminals reflects the total number of available synaptic vesicles, i.e. the pool size (according to EM analysis, 330 ± 27 at vitrified CA3-CA1 hippocampal synapses) (1). Most of the FM1-43 molecules inside synaptic vesicles are lost after they fuse again with the plasma membrane, with a destaining rate reflecting both the modality (full fusion and kiss & run) and the rate of exocytosis but also the off-rate of the dye from the membrane. Fractional destaining provides direct information on the number of vesicles released per stimulus and thus on release probabilities (Pr) with a signal to noise ratio that in some cases allows the investigation of individual synaptic vesicles (2, 18). One caveat of this technique is that the phase of FM1-43 uptake (i.e. the endocytosis of dye by synaptic vesicles) cannot be followed directly during the loading phase, due to the aspecific signal from dye incorporation in the plasma membrane. Specific double pulse protocols have been then developed to gather actual rates of vesicle endocytosis.

ANTIBODIES AGAINST INTRALUMENAL EPITOPES OF SYNAPTIC VESICLES

During exocytosis luminal components of synaptic vesicles are briefly exposed at the surface of presynaptic terminals. An ingenious experimental approach permits to

visualize exo-endocytosis by the cycling exposure of synaptic antigens. Vesicular fusion and recycling are detected by the uptake of specific antibodies directed against the luminal epitope of synaptic vesicle proteins, for example synaptotagmin-I (7, 11-13). Addition of these antibodies to the culture medium of neuronal cells results in their binding to exposed luminal epitopes followed by their internalization inside recycling synaptic vesicles as demonstrated by immunogold EM (13). These findings demonstrated very convincingly that it is possible to label synaptic terminals with a very specific tool based on an antigen-antibody interaction. Because of this binding specificity, the specific loading of antibodies in synaptic vesicles greatly exceeds the constitutive bulk flow uptake via endosomes even when challenging resting terminals. Thus with this approach there is no need to stimulate synaptic terminals as for the FM1-43 labeling protocol and therefore it is possible to visualize effectively spontaneous exo-endocytosis of synaptic vesicles (12). Another important advantage of this methodology is that antibodies are retained inside synaptic vesicles even after subsequent cycles of exo-endocytosis (12) and this greatly simplifies the labeling procedure since, without excitation, there is little loss of uptake signals during the course of an experiment. One caveat of both FM1-43 and antibodies labelling of synapses is the possible occurrence of transient modalities of exocytosis that do not proceed toward full fusion or synaptic and vesicle membranes. The importance and extent of this modality of exocytosis also known as kiss & run has been fully established at endocrine cells by Erwin Neher and coworker (7) but is not clear yet what is the actual scenario at brain synapses. In any case, the amount of synaptic staining with both FM1-43 and anti-synaptotagmin antibodies correlates with the number of quantal release as detected by standard electrophysiology (12) and so it can be used to estimate the number of vesicles released per stimulus and release probabilities (Pr). On these grounds, ratiometric approaches using the sequential uptake of two different sets of anti-synaptotagmin-I antibodies can be effectively used to monitor changes in presynaptic activity (12). In these experiments, the relative amounts of antibodies internalization can be assessed by using two different antibodies, either raised in two different animal species (Rabbit and Goat) and then detected by species-specific fluorescent secondary antibodies (indirect immunofluorescence) or by labelling the same anti-synaptotagmin antibodies with two different fluorophores (this permit on line measurements of synaptic uptake) (11). This simple ratiometric method provides a measure of changes in exocytotic activity following experimental interventions and allows each synapse to serve as its own control. In this way, synaptic modulation can be studied with great spatial resolution across complex dendritic arborizations. One important limitation of this ratiometric technique is that it assumes that the endocytotic process is not different in the two experimental epochs. For this reason, although a possible change in endocytosis could be tested with other techniques it is more correct to interpret these as changes in exo-endocytosis. This approach has been used to test the idea that presynaptic modifications contribute to long-term potentiation of synaptic transmission in CA3-CA1 hippocampal cultures (11) a result that has been also confirmed by FM1-43 uptake experiments by Siegelbaum and coworkers. We will now

describe in details some of the methodologies which are needed to use anti-synaptotagmin-I antibodies and FM1-43 for synaptic studies.

TECHNICAL ASPECTS OF MULTI-LABELLING IMMUNOFLUORESCENCE

After synaptic labelling with antibodies, these are usually detected by species-specific fluorescent secondary antibodies, a procedure known as indirect immunofluorescence. The main caveats of this approach come from the aspecific labeling of brain cells and brain tissue by antibodies and from their aspecific fluorescence or autofluorescence. The aspecific labelling usually arise from aspecific reaction of primary and/or secondary antibodies, while the autofluorescence is produced by either endogenous cellular components or by chemical fixation. For weakly fluorescing signals, such as those observed with synaptic assays, it is very important to investigate the possible sources of background fluorescence and to minimize their contribution. First of all, specificity and crossreactivity of the antibodies in use should be tested. This will include a western blot analysis of the affinity purified antibodies and preimmune sera with or without excess of antigen. Similar tests should be performed at the immunofluorescence level and in these experiments either the primary or the secondary antibody should also be omitted. Regarding secondary antibodies, it is always recommended to buy commercial fluorescently labeled secondary antibodies cross-purified across many different animal species. Chemical fixation produces free aldehydic groups on proteins that can greatly enhance aspecific binding of antibodies. For this reason it is very important to quench very well these groups with quenching agents. For multi-labelling experiments it is also very important to choose carefully the different fluorochromes and the properties of the excitation-emission filter sets to avoid bleed through between channels but also energy transfer between dyes. With a specific set of filters and fluorophores, the amount of bleed through can be easily determined by quantifying in all emission channels the synaptic fluorescence, when only one set of primary-secondary antibodies is used. Fluorescent energy transfer can be minimized by selecting fluorophores where there is the least overlap between the emission spectra of one with the excitation spectra of the other and by always using the longest wavelength emitting fluorophore as a reporter for the most abundant synaptic antigen. About the nature of cellular autofluorescence in unfixed conditions little is known. Nucleotides such as NADH, NADPH, riboflavin, FAD and fluorescent proteins such as lipofuscin are certainly among the contributing factors. Autofluorescence emission is stronger at 500-540 nm, in the range of fluorescein emission, and this is the characteristic wavelength of riboflavin and lipofuscin. Interestingly, in our primary neuronal cultures the spatial location of the autofluorescent signal allows us to draw a few important conclusions: i) most of the autofluorescence seems to arise from well-defined internal structures, i.e. endo-lysosomes, larger vacuoles and mitochondria while the nucleus always appears dark; ii) organelles located inside glial cells seem to be the most brightly fluorescing and thus, althou-

gh fewer in number, glial cells intermixed with neurons can produce a large fraction of the global autofluorescent signal. We should point out that often these fluorescent vacuoles (located in glial cells) can be as large as a few microns, and thus without additional controls they can sometimes be misinterpreted as synaptic varicosities; iii) cells are much more autofluorescent in living conditions than after chemical fixation and permeabilization with detergents. This suggests that a large component of the autofluorescence signal arises from freely diffusible compounds which are not directly linked to proteins, since chemical fixation doesn't immobilize them. In our experience, some of these fluorescent compounds must derive from ingredients present in the cell culture media that get accumulated inside cells. In particular, we have found that the pH indicator phenol red, which fluoresces on a broad range, enhances cell autofluorescence. Phenol red is a weak amine and thus, similarly to other analogous compounds such as acridine orange, it must be trapped in cellular compartments with low internal pH where it is retained due to protonation. This certainly contributes to the autofluorescence signal of endolysosomes and larger vacuoles of living cells since cultures maintained from day 0 after plating in a culture media devoided of phenol red display a reduced autofluorescent. Some of this signal might be retained after fixation-permeabilization possibly due to incomplete endolysosomal permeabilization but more probably because of chemical interaction of the dye with proteins.

FLUORESCENCE DETECTION FROM INDIVIDUAL SYNAPSES

How to collect and to quantify antibodies and FM1-43 fluorescent signals from labeled synapses? If we are willing to gather these signals from individual terminals, the essential piece of equipment consists of either a one- two-photon scanning laser microscope or a conventional fluorescent microscope equipped for video microscopy and digital image processing. It is not appropriate here to go into great detail of the theory of conventional and confocal microscopy. However, since one-photon confocal microscopy has become a very popular choice for synaptic studies, it is important to present at least some little theory about fluorescent measurements with scanning confocal microscopes in order to understand better the major advantages over conventional microscopes. The main difference between a confocal and a conventional fluorescent microscope is that image formation is obtained by probing the specimen point by point with a small source of light produced by a laser through a point aperture (a pinhole aperture). Detection is achieved through a point detector in the light path which is adjusted to be confocal with the point source of light. Which are the advantages and disadvantages of such an optical system?

In an image-forming system, resolution is a measure of the ability to separate images of two neighboring object points. In the absence of diffraction and aberrations, each point object would, accordingly to geometrical optics, give rise to a sharp point image. But when the light emitted by a point source passes through a perfect lens, light waves emerging from the back aperture interfere with each other and produce a 3D diffraction pattern centered around the focus of the lens. In the image plane,

this appears as a two-dimensional diffraction pattern (airy disk) with a characteristic bell-shaped intensity profile whose radius is inversely proportional to the numerical aperture (N.A.) of the lens according to:

$$r = 1.2 \lambda / 2 \text{ N.A.}$$

where λ is the wavelength of light and N.A. is given by:

$$\text{N.A.} = n \sin\alpha$$

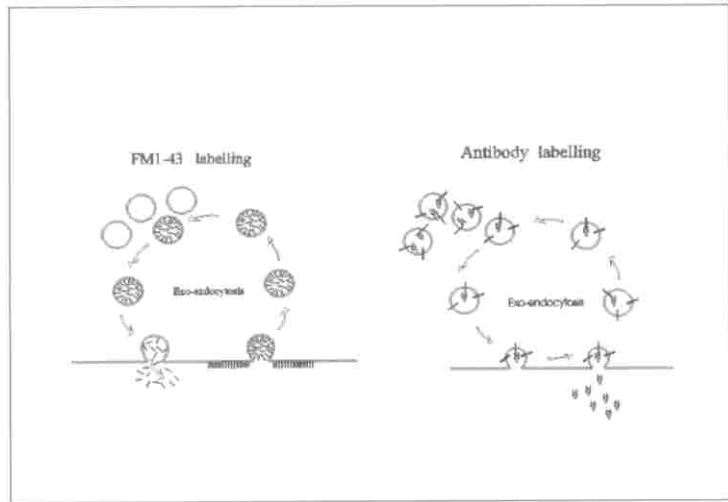
where n is the refractive index of the medium outside (the velocity of monochromatic light in the medium over the velocity in the vacuum) and α is the angle of light captured by the lens. In these conditions, according to the Rayleigh's criterion, two images are regarded as just resolved when the principal maximum of the diffraction pattern of one coincides with the first minimum of the other (peak distance $\geq r$). According to spatial frequency arguments, when the field of view becomes significantly smaller than the airy disk, as in confocal with small detection pinholes, the x-y resolution can be further increased of ~ 1.4 folds. But this is not the crucial advantage of this fluorescence imaging technique which derives most of its popularity from an impressive depth discrimination. The theoretical axial or z resolution in a microscope also depends on the tridimensional diffraction pattern which is more elongated in the z direction than in the lateral direction. For this reason the axial resolution is at most $\sim 50\%$ of the value achieved on the x-y axis and also since axial resolution rises with the square of the N.A., it is much more sensitive to this parameter than the lateral resolution. This value can be calculated according to the formula:

$$r_z = 2 \lambda n / \text{N.A.}^2$$

The important aspect is that this theoretical value for axial resolution is never achieved with conventional microscopy because of signal degradation by light arising from regions above and below the focal plane. In confocal microscopes, the presence of a limiting point detector ensures that we acquire only the fluorescent light originating from the illuminated spot with *reduced blurring* of the image from light scattering and background fluorescence from out of focus structures. This spatial filtering increases the effective depth discrimination which is best when the detector aperture is set to its minimum. In optimal conditions a resolving power of a fraction of a μm can be obtained (10) and this is an excellent value also considering that the diameter of a CNS synapse is in the order of $\sim 1\text{-}2 \mu\text{m}$ (1). Thus, defocusing does not create blurring, but gradually cuts out parts of the neuron as they move away from the focal plane. In this way, synapses positioned at different levels of a three-dimensional somatodendritic neuronal tree can be imaged individually and the whole structure can be reconstructed from stacks of images obtained at different focal planes.

Fig. 1. - *Cartoon illustrating the use-dependent uptake of dyes and antibodies.*

Specific markers such as anti-synaptotagmin antibodies are specifically trapped inside active terminals during cycles of exo-endocytosis (right). Since these antibodies are not released following exocytosis (12) their concentrations will steadily increase during successive cycles of exo-endocytosis. On the contrary, FM1-43 molecules (left), after being trapped inside synaptic vesicles can be discharged following exocytosis.



Another very useful advantage of scanning microscopes is the electronic control of magnification, i.e. the zoom. This is a very handy feature since it allows to change magnification without changing lenses. This procedure does not determine loss of contrast, detector bandwidth and gain with the advantage that it is possible to select the objective with the best N.A. thus increasing the effective resolution. Since with this procedure we work more nearer to the optical axis, a reduction in aberration is also expected (see below). Unfortunately, higher zoom means also that the number of photon hitting the sample will increase (as the square of the zoom setting) and strong light intensities have a lot of drawbacks (see below).

Labeling of the same synaptic structure using different dyes is very simple when we are using a confocal microscope since the multiline excitation mode of lasers is a very reliable source of differential excitation with high degree of monochromaticity. Although no one laser can provide all of the wavelengths needed to excite the most common dyes, the multiline mode of Argon-ion lasers (457, 488, 514 nm) and Argon-Krypton lasers (488, 568, 647 nm) represents a good compromise and allows the simultaneous use of three fluorophores with widely separated excitation spectra (FM1-43 is excited by the 488 nm line). As an alternative approach, when we are using fluorophores emitting at the same wavelength, in some experimental conditions it is also possible to label sequentially the same structure with two different dyes (for example FM1-43 and Fluo-3). So many advantages but also a few important caveats come into play when we use intense excitation such as those produced by laser sources. In this regard, light rejection through the pinhole means that less light will strike the detector and thus imaging will require significantly higher level of excitation to achieve a good resolving power. When a fluorophore molecule is exposed to such a high intensity excitation saturation, bleaching of the fluorophore and damage to the specimen are almost inevitable drawbacks and these limit the duration of the experiments. Accordingly, the best compromise is to combine an

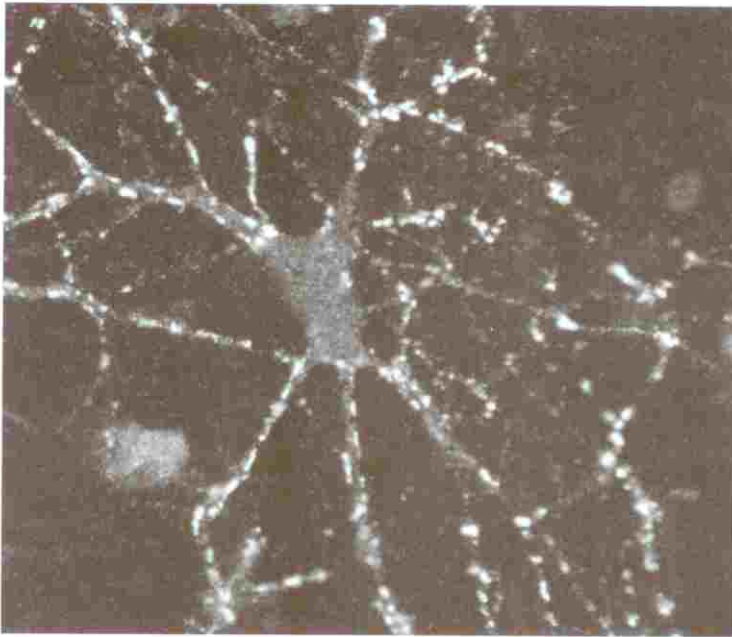


Fig. 2. - *Fluorescent picture of active synapses in CA3-CA1 hippocampal neurons.*

Synaptic terminals were visualized through the uptake of anti-synaptotagmin-I antibodies generated in rabbit. Living hippocampal neurons were incubated for 2 hours in regular culture medium (37 °C, 5% CO₂), with the addition of anti-synaptotagmin-I antibody and 1 μM TTX. The fluorescence spots represent active synaptic terminals. Note the variability in the extent of synaptic staining under control conditions reflecting different synaptic size and different degree of synaptic activity across the synaptic population. Bar, 50 μm.

improvement in light collection efficiency, including high N.A. objectives and high quantum efficiency photocathodes, with an increase in the fluorescent signal by enlarging the detector pinhole thus losing some spatial resolution.

When a fluorophore is excited, it enters into a different singlet electronic energy level from where it can relax to the original ground state due to a combination of radiation less internal conversion (which causes the Stokes shift, i.e. the difference between the emission and excitation peak wavelengths) and fluorescence emission. Since the overall process has a finite lifetime (excited state lifetime $\sim 10^{-6}$ - 10^{-9} s), there is a maximum rate at which we can get photons out from fluorophores and this is obtained when most of the fluorophore molecules have been shifted from the ground state toward the excited state. During intense excitation, an additional complication arises from inter system crossing via a spin flip which determines the trapping of fluorophores in the triplet state, a much more stable and long lived configuration. The latter process effectively reduces the concentration of active molecules (26). In these conditions, a further increase in excitation will not produce any increment in the light output but in contrast, since parasitic processes such as light scattering and autofluorescence do not saturate but increase linearly with increased excitation intensities, a drop in the signal-to-noise ratio of the scanned image should be expected.

In this context, bleaching of fluorophore molecules becomes an important factor with a noticeable decay of fluorescence intensity at each scan and the accumulation of bleached, unfluorescent molecules. These molecules can be produced from both singlet and triplet excited states although, more commonly, from molecules absorbed in the triplet state and oxygen must be somehow involved in this phenomenon

since antioxidant molecules have a protective effect (26). Since each fluorophore has a limited "working" lifetime (for fluorescein typically 20,000 photons before it loses its fluorescent properties), bleaching effectively limits resolution and the duration of the experiments. Furthermore the reaction of dye molecules with oxygen sensitizes the formation of reactive singlet oxygen and other free radicals. These reactive radicals attack membrane components and damage cell structures (photodynamic damage) and this is another important factor limiting the amount of excitation and the duration of the experiments on living synapses. Since excitation is not restricted to the image plane, bleaching and photodestruction take place in a conical volume above and below the structure in focus (9) and thus great care should be taken when acquiring data from stacks of images from different planes.

As clear from above, the focal length of an optical system changes with λ . In fluorescent confocal microscopy, where the excitation and emission lights have a different λ (the larger difference the bigger is the Stokes shift of the dye) axial and lateral chromatic aberration errors can displace the excitation spot from the collection point (more evident with small pinholes). This phenomenon causes a large reduction in collection efficiency: at 40-60 μm off axis a 50% signal reduction has been reported. The extent of this aberration will be mainly dependent on the aberration of the objective lenses and thus the lens elements must be corrected with respect to aberrations and flatness of field. However no matter how perfect these corrections are, aberrations will still appear when the microscope is used for biological samples which consist mostly of water, especially if the focal plane is far within this aqueous medium. This effect becomes more serious in multi-labelling experiments where dyes must emit at widely separated wavelengths. This is a potentially significant caveat since it may affect the validity of co localization data and it should be very carefully tested using for example small fluorescent beads imaged at different excitation λ . In these experiments it is important to mention that the aperture size should be identical in all channels to avoid comparing signals arising from different optical thickness.

IDENTIFICATION OF SYNAPSES AND FLUORESCENCE QUANTITATION

In order to quantify it is essential to be certain about the synaptic nature of the fluorescence spot. With FM1-43, it is important to acquire data only from fluorescence spots that stain and destain in an activity dependent fashion. With antibody labelling, it is essential either to use the ratiometric approach described above or to retrospectively stain fluorescent puncta for synaptic markers. Using the ratiometric approach, if either one of the two fluorescent signals from the same synaptic spot is below a pre-set threshold values (from noise distribution), the spot should not be analyzed. Thus, if the synapse is silent in either one of the two epochs, then it is excluded from analysis. The only way to get around this problem and monitor changes at silent sites is to label synaptic terminals with a third antibody, directed

toward the synaptic antigen that is used as a bate, such as the cytosolic portion of synaptotagmin. In this way synaptic terminals can be easily identified, even when one of the uptake measurements falls below the noise level. This additional synaptic signal can also be very important during data acquisition. In theory each group of synapses should be scanned only once to avoid different degree of bleaching of uptake signals among synapses but this is often impractical. With triple labeling this becomes possible since focusing and precise positioning of the sample can be obtained on the third channel (that does not reflect synaptic uptake but just the concentration of the antigen) without any bleaching of the other two channels. Regarding the actual measurements on synaptic envelopes, fluorescent intensities near the middle of individual boutons should be quantitated using a mask with a fixed profile, smaller than the actual size of the synaptic boutons. This permits to obtain values which are independent from bouton dimensions, hence from numbers of available vesicles. Since the assay is a ratiometric, no background subtraction should be performed to avoid over correction of intensity values, which would tend to give spuriously high values in ratio determinations. Similarly no digital filtering for edge enhancement and smoothing should be applied to prevent additional nonlinearities.

CONCLUSIONS

Besides the two approaches presented here, other techniques are becoming available such as chimeric synaptic proteins with fluorescent sensors for the occurrence of exocytosis (14, 15, 20) but also some advanced optical approaches such as evanescent microscopy combined with fluorescent staining of secretory vesicles (25) and measurements of failures rates of spine Ca^{2+} transients with one-two photon microscopy at individual spines (9).

Genetically encoded probes represent a great promise for the functional imaging of specified neuronal populations in the intact nervous system, yet their *in vivo* application has been limited. Synapto-pHluorin, a pH-sensitive protein reports synaptic vesicle fusion. Targeted expression of synapto-pHluorin in mouse will permit the analysis of previously inaccessible neuronal populations and chronic imaging from genetically identified neurons *in vivo*. To conclude, we must underline that a valuable goal in neurobiology is to arrive at a better understanding of how specific neuronal cell types, a specific subset of synapses perform in the *in situ* condition and this presupposes the ability to track synapses deep in the brain tissue. Unfortunately no quantitative method is yet available to directly visualize exocytosis and endocytosis in tissue explants or in the *in-vivo* brain and most of the techniques here described are applicable to neurons grown in culture, where synapses are more easily accessible for dye entry and for optical study. The hope is that in the near future, all this resurgence of interests and methods will lead to an efficient and non-invasive way to look directly at synaptic function in the *in vivo* brain.

SUMMARY

The two approaches presented here bypass postsynaptic receptors as indicators of quantal release, and thus they can provide information which is clearly distinct from that obtained with standard electrophysiological techniques. Indeed, the inherently variable responsiveness of the postsynaptic membrane makes it an unreliable indicator of presynaptic activity and this has fueled a lot of controversy, particularly in the area of synaptic plasticity. A major advantage of these two methods is their ability to detect changes at the single bouton level. This offers a lot of advantages including the possibility to study the functional role for exo-endocytosis but also plasticity against a background of great variability among a large number of synapses. The spatial resolving power of FM1-43 and anti-synaptotagmin antibodies may be valuable in future studies of spread of LTP between neighboring synapses and in the mapping the pattern of neuronal activity in complex networks of neurons.

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