

Optical Recording from Individual Neurons in Culture

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■ Introduction

Methods for optically recording dynamic processes in single living neurons must be considered in light of two fundamental questions: *what to record* and *how to record* it. Specifically, deciding *what to record* involves determining a parameter of interest (e.g., membrane potential or ion concentration), the nature of the information required (e.g., qualitative or quantitative) and the optical indicator best suited to making these measurements. Likewise, deciding *how to record* these signals involves consideration of recording methodologies (i.e., photometry or imaging), experimental procedures (e.g., loading and staining protocols) and data processing techniques (i.e., signal processing and analysis). Irrespective of which combination of methods are chosen it is important to understand the essential factors that contribute to obtaining high quality optical signals. By fully understanding the fundamental limits of this recording methodology, the novice investigator should be able to maximize signal quality and effectively solve any technical problems that might arise.

This chapter considers both instrumentation and experimental factors and their implications for making both qualitative and quantitative optical recordings from individual neurons in culture. In particular, appropriate methods for making fast recordings of various physiological parameters, with subcellular resolution, are documented. Two classes of indicators, voltage-sensitive dyes and calcium indicators, are used to illustrate the principles underlying successful optical recording. These principles can easily be extrapolated to other indicator types.

The scope of this chapter is limited to the consideration of methods for making physiological recordings from individual neurons or small group of cells in culture. Therefore, we do not consider methods used to examine fine structural details or localize cellular markers. Nor do we consider the cell culture methods necessary to produce neurons suitable for optical recording (instead, see Chapter 10). However, several cell culture properties that should be optimized to facilitate this kind of recording are documented. Optical recording methods for use in more complex tissues such as brain slices or in vivo preparations are considered in other chapters in this volume. In particular, the reader is referred to Sinha and Saggau (Chapter 16) and Grinvald et al. (Chapter 34).

There are a number of preliminary steps that must be undertaken prior to conducting an experiment employing optical recording methods. The remainder of this section examines several such issues. These include:

- Desirable properties of cell cultures for optical recording
- Methods for visualizing single neurons

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- Choice of indicator(s) (e.g. parameter, type of signal)
- Choice of recording technique (e.g. photometry or imaging).
- Choice of instrumentation (including light sources, optics and detectors).

Desirable Properties of Cell Cultures for Optical Recording

Neuronal cell cultures can take many forms depending on the cell type and culture conditions employed. Unfortunately, the conditions that favor good optical recordings are quite stringent and sometimes require modified culture methods. This is especially true in cases where indicators are bath-applied or subcellular resolution is required. In these cases the density of neurons or neuronal processes (i.e., axons and dendrites) should be sufficiently low to allow clear identification of the signal source. This is normally achieved by producing a single layer of cells at low density (e.g., ~ 200 cells per mm^2). Additionally, the number of non-neuronal cells in the culture (e.g., glia) must be kept to a minimum as they are also stained by optical indicators and can easily generate non-specific optical signals. Finally, cells should be cultured on a substrate optically compatible with high numerical aperture objective lenses and the contrast-enhancement techniques documented in the next section (i.e., $< 150\mu\text{m}$ thickness). In particular, while some plastic substrates have the preferred surface on which to grow some types of neurons, they must also be thin enough to accommodate the working distance of the objective lens. In addition, these substrates should be non-polarizing; otherwise they can disrupt image formation in cases where polarization-dependent contrast enhancement techniques are used (see below).

Methods for Visualizing Single Neurons

Single neurons in culture possess inherently low visual contrast. This is especially apparent with traditional brightfield illumination. In cell culture, phase contrast microscopy is commonly used to identify and assess the viability of these neurons. Typically, healthy neurons are very phase-bright, while unhealthy cells and glia are phase-dim. However, transillumination with phase contrast optics does not necessarily provide the high spatial resolution or the perception of depth appropriate to distinguish between cells or amongst small cellular structures. Hence other illumination methods are often used to select cells or parts of cells for optical recording. Some examples of these other methods are:

- Normarski or Differential Interference Contrast (DIC)
- Hoffman Modulation Contrast (HMC)
- Varel Contrast or variable relief contrast (VC)

While HMC is relatively inexpensive, easy to implement and insensitive to birefringence, its optical sectioning capabilities are typically less than DIC. However, DIC performs poorly with highly birefringent structures such as myelinated axons. Moreover, as DIC uses polarized light, care needs to be taken with plastic dishes or coverslips because their anisotropic nature can seriously degrade image quality. Varel relief contrast is a recent innovation and currently is available from only one vendor. Like HMC, VC is relatively low cost and easy to implement. However, it is more difficult to combine with epifluorescence optics. For more detailed information on these methods see Spector et al. (1998).

Choice of Indicator

One advantage of optical recording techniques is the variety of different physiological parameters (e.g., ion concentration, membrane potential and second messengers) that can be examined. However, for each of these parameters there are a vast array of seemingly similar indicators to choose from. For instance, within each indicator class, a range of dyes are available based on a number of properties. These properties can include:

- Mode of application (bulk loading vs. single cell);
- Spectral properties (UV vs. visible excitation);
- Absolute binding affinities (for ions or membranes);
- Special properties (e.g., near-membrane dyes or low-leakage or dextran conjugates).

Prior to undertaking studies using optical indicators a number of important choices must be made regarding the indicator to be employed. These decisions are typically based on the underlying experimental objectives and the likely signal characteristics. For instance, an investigator must decide whether a qualitative result is sufficient or a quantitative result is required and therefore ratiometric methods should be employed. To aid the reader in this decision making process a simple flowchart is presented below (Figure 1.).

Other more advanced considerations (e.g., *sensitivity, brightness, and photostability*) that are important in choosing between these seemingly similar indicators are discussed in a later section (“Criteria for Comparing Indicators”). Two excellent sources of information about currently available optical indicators are Haugland (1996) and Johnson (1998).

A variety of optical parameters can be measured and related to a physiological variable of interest. For instance, some indicators require that fluorescence be monitored while others are best assayed with absorbance methods. In some instances, both fluorescence and absorbance can be measured from the same indicator. Still other indicators are luminescent and require no excitation at all.

Quantitative vs. Qualitative Indicators

Fluorescence vs. Absorbance

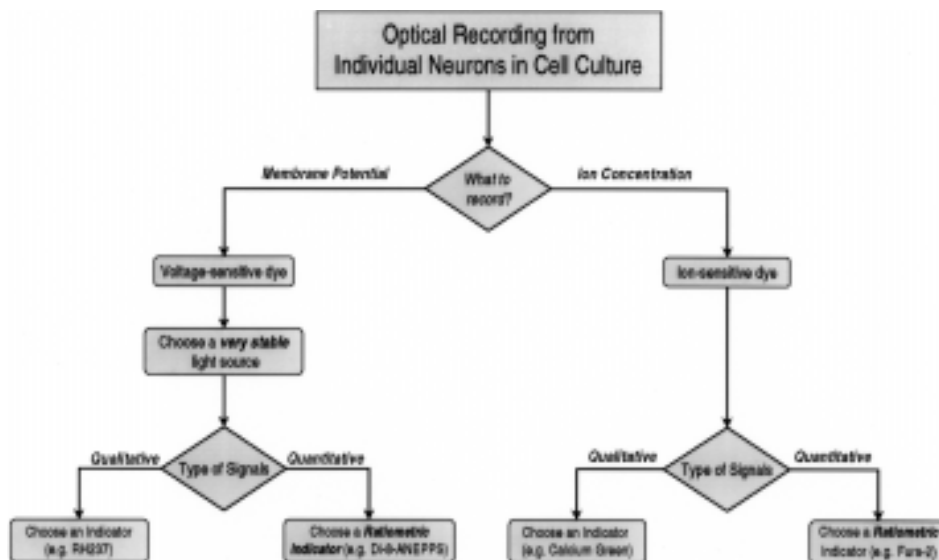


Fig. 1. Decision making scheme to determine “What to record”.

In general, when optical signals are assayed from small structures or when a low concentration of indicator molecules is used, fluorescence methods are preferred. This is especially true in experiments utilizing cell cultures where fluorescence methods are almost always favored over other possibilities. In this case, fluorescent signals are usually superior to those recorded with absorbance approaches because of the limited surface area and/or volume of subcellular structures from which the signal is generated. Furthermore, the signals from fluorescent dyes ($\Delta F/F$) are typically larger than corresponding signals ($\Delta I/I$) from absorbance indicators and thus the instrumentation requirements are less rigorous. In light of these facts, subsequent discussions are limited to fluorescent indicators.

The choice of optical indicator will, in part, dictate which light sources, optical configuration and detector(s) are employed. Different classes of optical indicators exhibit different characteristics and require optimization of different parameters. In this chapter we have chosen to examine two fundamentally different kinds of fluorescent optical indicators: *voltage-sensitive dyes* and *Ca indicators* (see below). Each of these indicators presents a distinctly different set of instrumentation requirements and methodological problems.

Voltage-sensitive Dyes

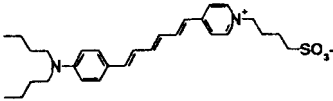
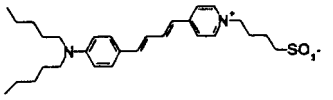
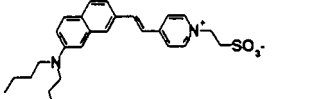
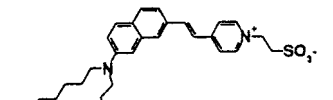
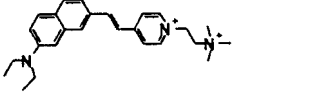
Optical indicators that are sensitive to membrane potential are commonly referred to as *voltage-sensitive indicators* or *voltage-sensitive dyes (VSDs)*. Two classes of VSDs are recognized based on their response time to changes in membrane potential. They are referred to as either *slow-response VSDs* or *fast-response VSDs*. Slow-response VSDs are also called *redistribution dyes*. Typically these membrane-permanent dyes partition between extracellular and intracellular compartments in a manner dependent on membrane potential. In general, redistribution dyes exhibit high sensitivity to membrane potential ($\Delta F/F > 10^{-2}$ per 100mV). However, the slow response time of these dyes (usually in the second range) caused by their electrodiffusion across the membrane, strongly limits their usefulness for measuring neural activity. Fast-response VSDs are typically amphipathic and *membrane-impermanent* dyes that attach to membranes and change either their orientation or parts of their structure in response to a change in the electrical field. Such structural changes cause these molecules to exhibit a change in the *fluorescence*. In general, fast-response VSDs show quite small changes in fluorescence with respect to a change in membrane potential (10^{-4} - 10^{-2} per 100mV). However, these indicators have fast response times (usually in the μ sec range) which make them attractive for measuring neural activity. Except for some dyes that exhibit voltage-dependent spectral shifts, the absolute calibration of fast-response VSD signals is difficult. Dyes exhibiting voltage-dependent spectral shifts are commonly designated *electrochromic* and allow ratiometric measurements and therefore absolute calibration.

Deciding between different VSDs within the same class is a difficult task. In addition to differences in absolute voltage-sensitivity and brightness, most VSDs exhibit species and cell type differences in voltage sensitivities and membrane affinity (Ross and Reichardt, 1979). Often the best voltage-sensitive dye for a particular application is determined empirically. Some of the most widely used fluorescent VSDs for single cell studies are documented in Table 1.

Ca Indicators

Most modern *calcium-sensitive dyes (CaSD)* are *tetracarboxylic dyes* which were derived from the *calcium buffer* BAPTA. In fact, a large family of fluorescent calcium indicators has been created by conjugating BAPTA with different fluorophores. Members of this family, which includes Fura-2 and Fluo-3, are highly selective for calcium over other cations. Two types of measurements are typically made with these modern calcium indicators: qualitative or quantitative. Different indicator molecules are required for each type of measurement. Qualitative measurements reflect changes in calcium levels without

Table 1. Fluorescent Voltage-sensitive Dyes Used in Single Cell Studies

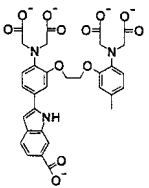
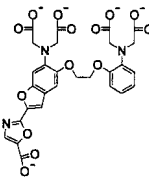
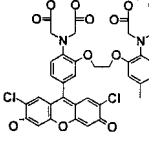
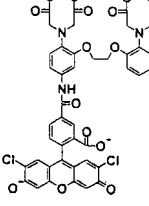
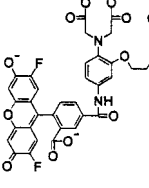
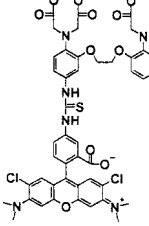
| VSD | Site of Application | Structure | Signal Size (per 100 mV) | Relative Membrane Affinity | Refs. |
|-------------|---------------------|--|--------------------------|----------------------------|-------|
| RH 237 | Extracellular |  | 2 % | Low | 1, 2 |
| RH 421 | Extracellular |  | 5 % | Moderate | 3 |
| di-4-ANEPPS | Extracellular |  | 10 % | Moderate | 4 |
| di-8-ANEPPS | Extracellular |  | 20 % | High | 5, 6 |
| di-2-ANEPEQ | Intracellular |  | 3 % | Low | 7,8 |

Pine (1991a) (2) Chien and Pine (1991b), (3) Meyer et al. (1998), (4) Kleinfeld *et al.* (1994), (5) Rohr and Salzberg (1994), (6) Bullen and Saggau (1997) (7) Antic and Zecevic (1995), (8) Zecevic (1996).

reference to resting levels or the absolute size of these changes. This kind of measurement is normally depicted as the change in fluorescence normalized by the overall mean fluorescence ($\Delta F/F$). In contrast, quantitative measurements are made ratiometrically and give an estimate of absolute calcium changes. Ratiometric measurements are particularly useful because they inherently eliminate distortions caused by photobleaching, variations in probe loading and retention, and by instrumentation factors such as long-term illumination instability. However, this kind of measurement typically requires a post-experiment calibration. Commonly, indicators employed for ratiometric determinations undergo calcium-dependent spectral shifts while qualitative indicators simply change their brightness in proportion to bound calcium. CaSDs are also distinguished by their *binding affinity* and *relative sensitivity*. The *binding affinity* indicates their sensitivity for Ca ions and is described by the dissociation constant (K_d). In contrast, the *relative sensitivity* indicates the magnitude of their fluorescence change to fluctuations in calcium concentration. This parameter is normally given as a ratio of calcium-bound and calcium-free levels. When compared to VSDs, calcium indicators typically produce a much larger change in $\Delta F/F$ (i.e., 10^{-2} - 10^0) and consequently are less affected by source noise (i.e., variations in illumination intensity from the light source that are directly reflected in the resulting fluorescence).

Some fluorescent CaSDs commonly used in single cell studies are documented in the adjacent table (Table 2.).

Table 2. Fluorescent Calcium-sensitive Dyes Used in Single Cell Studies

| CaSD | Ratiometric | Structure | Ca Affinity (K_d) | Relative Sensitivity | Refs. |
|----------------|-------------|---|--|--------------------------------------|-------|
| Indo-1 | Emission |  | Std. - 230 nM 1EF - 33 μ M | R_{max}/R_{min} 20 20 | 1, 2 |
| Fura-2 | Excitation |  | Std. - 145 nM 2FF - 35 μ M | R_{max}/R_{min} 45 45 | 1, 2 |
| Fluo-3 | No |  | Std. - 390 nM 3FF - 41 μ | F_{Ca}/F_{Free} 200 120 | 2, 3 |
| Calcium Green | No |  | 1N - 19 nM 2N - 550 nM 5N - 14 μ M | F_{Ca}/F_{Free} 14 100 38 | 2, 4 |
| Oregon Green | No |  | 1-170 nM 2 - 580 nM 5 - 20 μ M | F_{Ca}/F_{Free} 14 100 44 | 2 |
| Calcium Orange | No |  | 1N - 185 nM 5N - 20 μ M | F_{Ca}/F_{Free} 3 5 | 2, 4 |

Molecular structures shown refer to Indo-1(Std), Fura-2(Std), Fluo-3(Std), CaGn-1N, OrGn-488-BAPTA-2 and CaOr-5N. (1) Grynkiewicz et al. (1985), (2) Haugland (1996) (3) Minta et al. (1989), (4) Eberhard and Erne (1991).

Dual Indicator Studies

In principle, measurements with two or more optical indicators could be made simultaneously from the same tissue using two or more dyes. However, simultaneous recording of two or more physiological signals from the same point in space and time is considerably more difficult than examining a single parameter alone (for details see Bullen and Saggau, 1998 and Morris, 1992).

Choice of recording technique

A number of recording techniques are available to obtain optical measurements from single neurons. Typically, an investigator must choose between some form of photometry and imaging. In pure photometry, a continuous measurement is made from the whole field or a subset of the field defined by a fixed aperture. While in imaging approaches, the signal is included in a series of images recorded at equally spaced intervals in time. Different variations of these basic techniques are possible and some of the more common types are depicted schematically in Figure 2.

Each of these approaches possesses various advantages and disadvantages depending on the experimental objectives. A comparison of these approaches and their compatibility with various types of optical indicators is documented in the table below (Table 3).

In its simplest form, photometry involves a single measurement from a predefined area. As shown in Figure 2, this area can encompass a whole cell, parts of a single cell or parts

High-speed, random-access, laser scanning microscopy

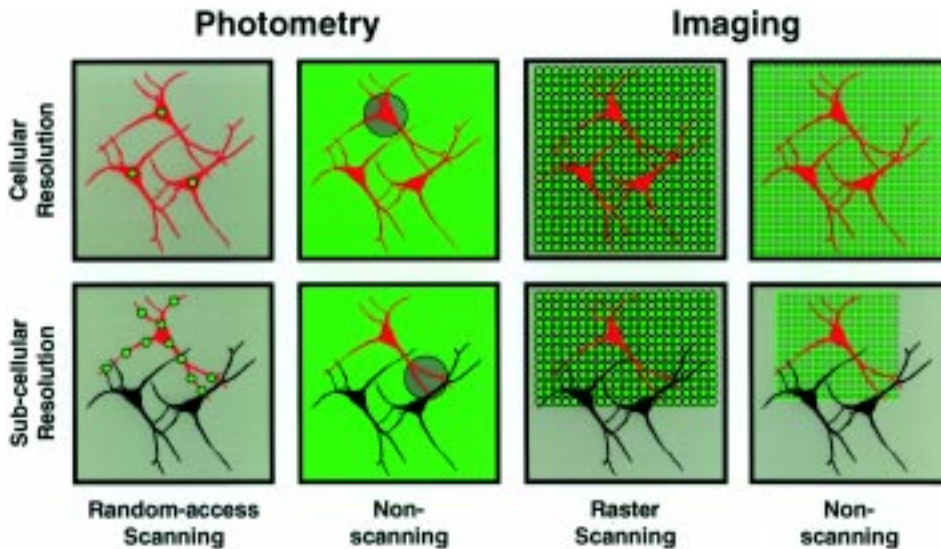


Fig. 2. Different types of Optical Recording Schemes Used in Single Cell Studies. Note: the cell(s) stained with optical indicator are always shown in red.

Table 3. Comparison of Optical Recording Methods for Use with Single Neurons

| | Photometry | | Imaging | |
|-----------------------|--|---|--|---|
| | Non-scanning (e.g., PMT or single photodiode) | Scanning (e.g., random access scanning microscopy) | Non-scanning (e.g., imaging detector) | Scanning (e.g., confocal microscopy) |
| Spatial Resolution | None | High | High | High |
| Temporal Resolution | High | High | Low to moderate | Low |
| Compatible with CaSDs | Yes | Yes | Yes | No |
| Compatible with VSDs | Yes | Yes | No | No |

of multiple cells. The obvious advantage of this approach is recording speed which can be very high. However, simple photometry only provides information from one site or area at a time. An alternative form of photometry with spatial resolution is “scanning photometry.” In this approach optical recordings are made from multiple interlaced recording sites with a scanning light source. One implementation of scanning photometry is “high-speed, random-access, laser scanning microscopy”. In this composite approach image capture and optical recording function are performed separately to gain temporal bandwidth and/or spatial resolution. Through the use of a very fast scanning scheme based on acousto-optical deflection this method repeatedly samples a series of predetermined scanning sites, with high digitizing resolution and at rates compatible with the fastest physiological events. Thus, this approach is able to optimize both spatial and temporal resolution. For more details about this approach see Bullen et al. (1997).

Imaging applications can also be divided into scanning and non-scanning classes. Non-scanning approaches typically use a scientific grade video camera (i.e., cooled CCD) or a lower spatial resolution photodiode array. The spatial resolution of these systems is dependent on the number of pixels per dimension which can be quite high (i.e., 1024). However, the drawback of many of these systems is their poor temporal resolution. Moreover, the full range of spatial resolution cannot always be utilized because spatial averaging techniques such as binning are often required to generate a useful physiological signal.

An alternative to camera-based imaging systems is various forms of scanning *microscopy*; in particular, *confocal microscopy* and *multi-photon microscopy*. Many types of confocal microscopes are commercially available and are often considered for optical recording from single neurons. In comparison, two-photon microscopy is a relatively recent innovation and there are still only a few systems in existence. Both these technologies allow imaging in complex three-dimensional preparations. However, several fea-

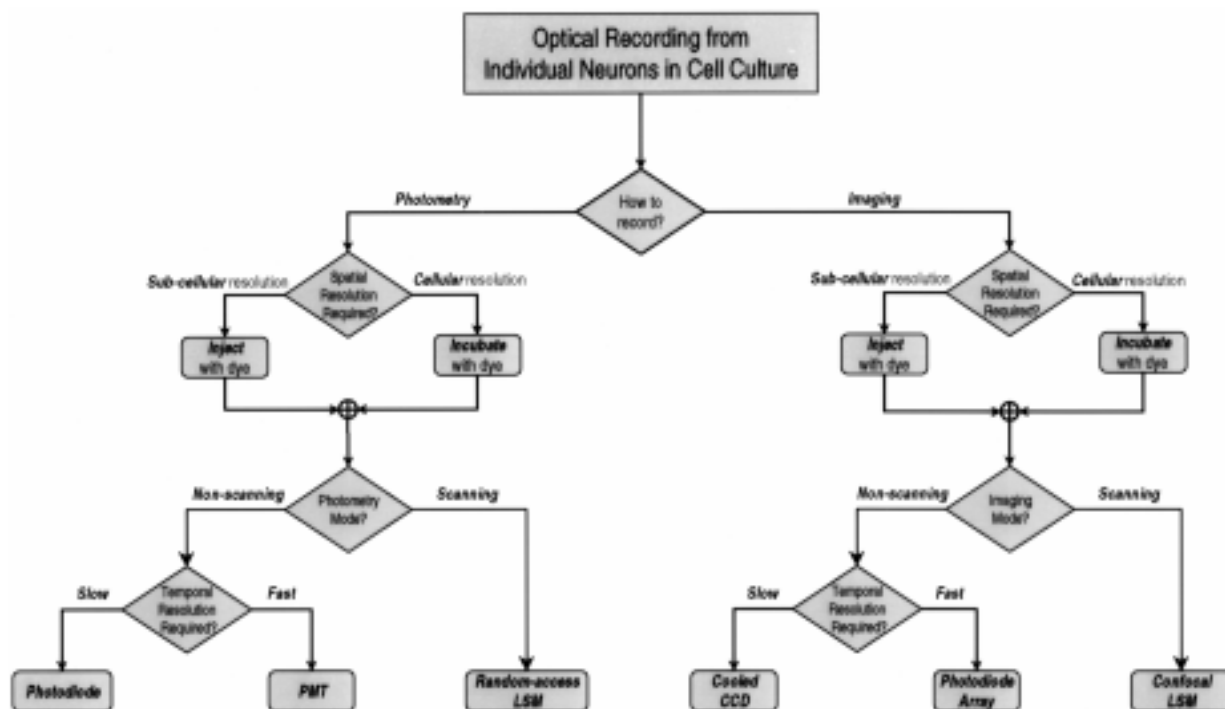


Fig. 3. Decision making scheme to determine “How to record”.

tures of these instruments limit their suitability for optical recording from single neurons. Most obviously, these instruments are almost exclusively imaging devices with limited overall frame rates and therefore they are not very useful for recording fast physiological signals. Moreover, commercial versions of these instruments commonly employ only 8-bit digitizing resolution, which is sometimes sufficient for CaSDs but is generally insufficient for use with VSDs. Finally, the considerable cost of these instruments is a significant issue for the novice investigator. Furthermore, because cultured neurons are typically a monolayer of cells, there is little benefit to be gained by adding an optical sectioning capability or from sub-micron spatial resolution. To aid the reader in deciding between this array of recording possibilities the following flowchart was derived to simplify the process (Figure 3).

Instrumentation

Correct instrumentation choices are important. In particular, it is critical to optimally illuminate the preparation to ensure success. Furthermore, collecting the maximum amount of emission light available ensures the highest possible signal quality. These instrumentation choices include consideration of:

- Light sources
- Optics
- Photodetectors

Important factors in determining the best light sources include:

- Brightness
- Spectral distribution
- Intensity stability

The most common light sources for optical imaging are:

- Tungsten halogen bulbs
- High pressure gas discharge bulbs
- Lasers

Tungsten halogen bulbs exhibit the highest intensity stability ($\Delta I/I = 10^{-5}$ - 10^{-4}). The spectrum of tungsten bulbs can be regarded as mostly white light, with a weak emission in the UV. *High-pressure gas discharge bulbs* such as Hg- or Xe-burners are light sources with much higher intensity but their amplitude noise is somewhat larger (i.e., 10^{-4} - 10^{-3}). While Hg-burners emit an inhomogeneous spectrum with many peaks down to the UV, the spectrum of Xe-burners is quite homogeneous. If Hg-burners are chosen for fluorescent applications, care should be exercised to ensure that the absorbance spectra of the fluorophores and the associated excitation filter correspond to a known Hg emission line (e.g., 365, 405, 436, and 546 nm). *Lasers* are an increasingly attractive light source. In particular, their ability to generate a diffraction-limited illumination spot makes them a perfect high intensity light source for various scanning applications. Unfortunately, the relative noise of lasers is commonly quite high, usually in the 10^{-3} – 10^{-2} range. However, some recently introduced lasers with improved cavity designs have performed much better in this regard (i.e. 10^{-5} - 10^{-4}). In addition, the coherence of laser light can give rise to interference-based speckles that adds to the total noise, thus making the use of lasers for absorbance measurements difficult. Lasers inherently emit monochromatic light but only at a discrete number of wavelengths (or lines). Thus, if a laser source is to be used, indicators must be chosen that match the available laser lines.

Optical considerations include:

- Type of microscope (upright or inverted)
- Objective lens and condenser

Light Sources

Optics

- Filters
- Dichroic mirrors

I. Type of microscope: The type of microscope that is typically considered for optical recording from single neurons could in principle be either upright or inverted. However, inverted microscopes are commonly chosen because they allow the use of high magnification, high numerical aperture objective lenses.

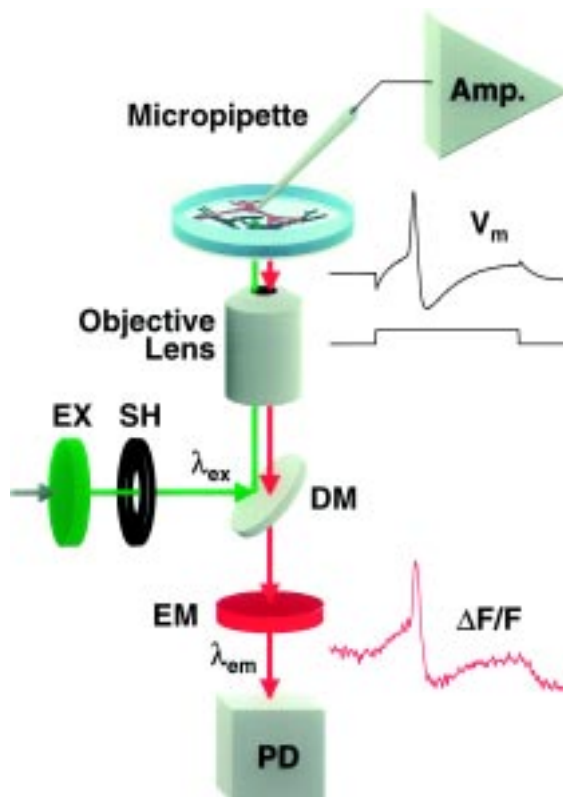
Likewise in fluorescence applications, an epifluorescence configuration is normally chosen over transfluorescence. There are several logical reasons for this choice. Firstly, with epifluorescence illumination, the excitation light and emitted fluorescence travel in opposite directions and are easily separated by a dichroic beam splitter. Secondly, the objective also serves as the condenser, assuring perfect alignment and maximal illumination and collection efficiencies. Epifluorescence illumination in combination with an inverted microscope also allows easy access with micropipettes. Finally, epifluorescence illumination is most easily combined with other transmitted light techniques such as phase contrast or DIC. A typical epifluorescence-recording configuration demonstrating these aspects is shown in Figure 4.

II. Objective lens and condenser: The objective lens is perhaps the most critical component in any microscope. It governs, among other things, the resolution, magnification and light gathering capabilities of the total system. In choosing an appropriate objective for optical recording from single neuron in culture the following features are critical:

- Magnification
- Numerical aperture
- Working distance
- Optical corrections

Fig. 4. Typical Optical Recording Configuration with Epifluorescence Illumination as Implemented on an Inverted Microscope.

EX = excitation filter,
SH = shutter,
 λ_{ex} = excitation wavelength,
DM = dichroic mirror,
EM = emission filter,
 λ_{em} = emission wavelength,
PD = photodetector,
Amp. = amplifier



Magnification: The magnification required to accurately measure fluorescence from a single neuron or parts of individual cells with spatial resolution corresponding to the structures of interest is typically 40x. Other commonly used objective lenses include 63x or 100x. It is important to remember that any additional magnification secondary to the objective, while increasing image size, does not add resolution or light gathering capability and therefore should be considered “empty magnification”.

Numerical aperture: In addition to its resolution, the numerical aperture (N.A.) of an objective lens determines its collection efficiency and hence the image brightness. Image brightness is proportional to the *brightness of illumination* and the *light-gathering power of the objective*. Both parameters are determined by the square of the N.A. of the lens and therefore in an epifluorescence configuration there is a 4th power relationship between image brightness and N.A. As a consequence, a relatively small change in numerical aperture can significantly change image brightness and thus signal strength in optical recording.

Working distance: Working distance (W.D.) is an important parameter listed in the specification of all lenses and refers to the free space between an *objective lens* and the specimen. It becomes a major issue with upright microscopes when imaging approaches are combined with micropipette applications. W.D. specifications are normally given by manufacturers as the distance above a normal thickness coverslip (#1). W.D. is inversely proportional to numerical aperture and thus the resolving power and collection efficiency of high N.A. lens normally come with a W.D. penalty.

Optical corrections: High quality objective lenses are commonly corrected for spherical and chromatic lens aberrations. While many of these corrections are important in image formation, they are in large part inconsequential for optical recording where other factors are limiting and most manufacturers have developed objectives specialized for fluorescence recording. However, it is important for an investigator to know the limitations of the various objective lens choices and understand the specifications given for each lens type.

Another important factor in choosing an objective lens is whether it is designed to act as a fixed tube-length or infinity-corrected lens. A fixed tube-length objective lens directly projects a real image within the microscope, normally at 160mm from the back focal plane. In contrast, an infinity-corrected lens projects this image towards infinity. The advantage of an infinity-corrected lens arises from the reduced number of subsequent relay lenses required in the optical path of a typical compound microscope. Fewer lenses in any given light path yield fewer internal reflections and higher relative transmission. This is especially true in cases such as DIC microscopy or epifluorescence microscopy where additional prisms or filters are inserted into the optical path.

Typically all these parameters are best met with high power, high N.A., immersion lenses specialized for fluorescence recording. Each microscope vendor offers one or more objective lenses that are specialized for recording fluorescence. Currently we use the Zeiss Fluor 100x (N.A. 1.3) objective but many other compatible lenses are available from Nikon, Leica, Olympus, etc. The condenser is of no practical importance for epifluorescence microscopy. However, it plays an important role in image formation with transillumination and for this reason, the condenser selected should be closely matched to the numerical aperture of the objective lens. This ensures that the resolving power of the objective is not limited by that of the condenser.

III. Filters and dichroic mirrors. The spectral properties of filters (i.e. excitation and emission) and the dichroic mirror(s) determine:

- The strength and appropriateness of excitation light.
- The relative separation between excitation and emission light.
- The collection efficiency for emission light.

Together these factors are strong determinants of signal strength and therefore signal quality. Under ideal conditions the excitation and emission filters (also known as exciter and emitter) chosen should be centered on the dye's respective absorption and emission peaks. To maximize the signal strength it is advantageous to employ filters that also have wide bandwidths. However, this strategy requires a sufficient separation between the excitation and emission spectra of the dye; otherwise optical cross-talk can occur between channels. A commonly used alternative is to employ a relatively narrowband excitation filter, because source light is plentiful and can be easily increased. Single laser lines are also an attractive excitation source for the same reason. The extra spectral separation achieved in these cases can then be used by the emission filter which should be as wide as possible to ensure all emission light is collected. In most cases the spectral properties of fluorescent indicators used in optical recording from single neurons are well characterized and standard filter sets are commonly available. A selection of these are documented in the following table (Table 4.). In special cases where novel filter designs are required, several manufacturers, notably Chroma or Omega (both of Brattleboro, VT), are proficient in producing customized optical elements.

Note: In some cases the signal size ($\Delta F/F$) of selected VSDs can be made larger by employing excitation and emission filters that do not correspond to the spectral maximum of the dye. This phenomenon arises from the voltage-dependent spectral shifts that occur in addition to the absolute amplitude changes caused by depolarization. For example, while the excitation and emission peaks of di-8-ANEPPS are 476 nm and 570 nm, respectively, Rohr and Salzberg (1994) found the best signal-to-noise ratio with this dye could be achieved with a 530(25) exciter, a DCLP560 dichroic mirror and a OG570 emission filter.

Table 4. Filters, Dichroic Mirrors and Laser Lines for Fluorescent Dyes Commonly Used in Single Cell Studies.

| Dyes | | Excitation (nm) | | | Dichroic Mirror | Emission (nm) | |
|------------------------|----------------|-----------------|--------------------|---------------|------------------|---------------|------------------------------|
| | | Peak | Filters (FWHM) | Laser Lines | | Peak | Filters (FWHM) |
| Calcium Sensitive Dyes | Indo-1 | 346 | 350 (20) | 351, 354, 355 | DCLP379, DCLP455 | 401, 475 | 400 (40), 480 (40) |
| | Fura-2 | 340, 380, (363) | 340 (20), 380 (20) | 334, 364 | DCLP430 | 512 | 510 (60) |
| | Fluo-3 | 503 | 490 (20) | 488 | DCLP505 | 525 | 525 (30) |
| | Calcium Green | 506 | 490 (20) | 488 | DCLP505 | 530 | 530 (40) |
| | Oregon Green | 496 | 490 (20) | 488 | DCLP505 | 524 | 530 (40) |
| | Calcium Orange | 549 | 540 (20) | 532 | DCLP560 | 575 | 580 (30) |
| Voltage-Sensitive Dyes | RH 237 | 506 | 500 (40) | 488, 514 | DCLP560 | 687 | OG610 |
| | RH 421 | 493 | 500 (40) | 488 | DCLP550 | 638 | OG590 |
| | di-4-ANEPPS | 476 | 470 (40) | 476, 488, 514 | DCLP570 | 605 | OG570 |
| | di-8-ANEPPS | 476 | 470 (40) | 476, 488, 514 | DCLP565 | 600 | OG570 or 540 (60) & 600 (60) |
| | di-2-ANEPEQ | 497 | 500 (40) | 488, 514 | DCLP575 | 640 | OG570 |

A substantial role in the overall performance of an optical recording system is played by the photodetector employed, and this component should be selected carefully. Detection devices can range from a single element photodetector used in the simplest forms of photometry to high resolution, scientific-grade cooled CCD cameras used in advanced imaging applications. In deciding between different detector types the main parameters of concern are:

- Sensitivity
- Quantum efficiency (QE)
- Dark noise
- Dynamic range
- Spectral response
- Cost

In imaging detectors, *readout speed* and *digitizing resolution* are also important considerations.

I. Non-imaging detectors The most prevalent single element photodetectors are *photodiodes* and *photomultipliers*. Such detectors have no spatial resolution but are still important for many imaging applications. For instance, in scanning microscopy, where an image can be produced with point illumination by sequentially scanning a whole preparation or parts thereof. Semiconductor *photodiodes* are an attractive photodetector option due to their high dynamic range, high quantum efficiency (>90%) and low cost. Despite their somewhat higher complexity and cost, *photomultiplier tubes* (PMT) are particularly attractive for scanning applications because they possess both sensitivity and response speed. These devices are an integral combination of a vacuum photodiode and a multistage photocurrent amplifier that makes use of multiple amplification stages to generate secondary photoelectrons. While the internal gain of a photomultiplier can be quite high (the gain increases exponentially with the number of stages), the true quantum efficiency is much less when compared to a semiconductor photodiode (about 10%, meaning that only every 10th photon generates a photoelectron). In addition, this internal gain also amplifies the dark noise. In comparing photodiodes and PMTs, it is somewhat difficult to make a global statement about which detector is better because it depends on the amount of light to be detected and the bandwidth required. Above a certain light level, the photodiode will always outperform the PMT and will only be constrained by shot noise limitations. However, below this level, the dark noise of the photodiode and its accompanying electronics will become dominant and the PMT will produce a better signal-to-noise ratio. This general principle is confounded by bandwidth considerations. The response speed of the photodiode at low light levels is limited by the time constant imposed by the large feedback resistors (i.e., G Ω range) required in the current-to-voltage conversion process. This is not a problem for the typical PMT because of the current amplification in each internal gain stage. Thus, in the case where both sensitivity (i.e., low light levels) and speed (i.e., scanning applications) are required, the PMT is probably superior to the photodiode.

II. Imaging detectors Various types of *video cameras* are the most common imaging detectors for optical recording. However, due to the requirements imposed by both the optical indicators and the speed of the signals to be measured, only very few imaging detectors are really suited to record neural activity. A normal video camera provides 30 frames/second and has a maximal intensity resolution of ~0.5% which is insufficient for optical imaging of neural activity with VSDs that requires a detector that supplies 10³ frames/second and the ability to resolve signals that are 10⁻⁴ of the static light intensity. One example of a scientific grade imaging camera that is often used in neurobiology is a frame-transfer cooled CCD camera. This device possesses high sensitivity, low

noise but only modest frame rates. A more appropriate imaging detector for recording fast neuronal activity is the low resolution *photodiode matrix (PDM)*. This photodetector can be regarded as an *array of single photodiodes*. Consequently, the favorable quantum efficiency and high sensitivity of photodiodes also applies to the photodiode matrix. The array sizes in these devices vary from 5x5 to 128x128 elements, with 10x10 or 12x12 being the most common. For very fast applications true *parallel access* can be used with arrays of up to 32x32 elements. Each photodiode is connected to its individual *current-to-voltage converter*, which can allow for gap-free recording (i.e., no shift or readout time delay). As was the case with a single photodiode, the bandwidth of such imaging detectors depends largely on the amount of light and the required signal-to-noise ratio. For a more detailed discussion of these issues see section “Data Acquisition and Digitization Issues”.

The following table (Table 5.) summarizes the relative merits of each photodetector.

Table 5. Comparison of Photodetector Properties

| | Sensitivity | Quantum efficiency | Dark noise | Dynamic range | Readout speed | Spatial resolution | Cost |
|------------------------------|-------------|--------------------|------------|---------------|---------------|--------------------|--------|
| Photodiode | + | +++ | - | +++ | N / A | 1 | \$ |
| Photomultiplier | +++ | + | -- | ++ | N / A | 1 | \$\$ |
| Photodiode matrix | + | +++ | - | +++ | +++ | 16x16 | \$\$\$ |
| CCD (cooled, frame-transfer) | ++ | ++ | --- | ++ | + | 512x512 | \$\$\$ |

Note: Quantum efficiency is defined as the ratio of photons detected over total number of incident photons. Sensitivity is defined as a measure of the minimum amount of detectable light.

■ Outline

The important steps in undertaking optical recording from single neurons can be divided into three parts:

- Instrumental design and construction
- Experimental design and implementation
- Signal analysis and presentation

Most of the instrumental considerations were addressed in the previous section and outlined in the flowcharts in Figure 1 and 3. The following sections document important considerations for the remaining two areas.

■ Materials

Cells: Previously prepared and plated at an appropriate density (i.e. 200–300 cells per mm²).

Solutions: Physiological saline(s) and drugs depending on experimental objectives.

Optical Indicators: Stock solutions ready for each specific application. Optical indicators are available from a number of sources with the best being Molecular Probes (Eugene, OR). See Haugland (1996).

Optical Recording System: Including microscope, light source, filters, epifluorescence optics and detector(s).

Auxiliary Electrophysiological Equipment: Such as stimulators, amplifiers, perfusion system and micromanipulators (cf. Chapter 5).

Data Acquisition System: Including computer, A/D and D/A plug in boards and data storage devices (cf. Chapter 45).

■ Procedure

This section addresses a number of methodological issues or experimental techniques important for successful optical recording. No single *procedure* is presented because experiments using optical recording methods can be quite heterogeneous in nature. Instead, those elements that are common to all experiments are considered. Many of these elements are quite mundane but often represent the difference between successful experiments and unnecessary frustration. These considerations include:

- Mixture and storage of optical indicators
- Loading and staining protocols
- Experimental design issues
- Calibration procedures
- Signal processing methods

Mixture and Storage of Optical Indicators

There is no universal way to solubilize and store these indicators. In most instances the optimal conditions are empirically determined for each dye. Because of their amphipathic nature most VSDs are not inherently water-soluble and external agents are sometimes required to solubilize them. In addition, other agents are sometimes required to aid in partitioning these dyes into membranes. Various solvents or combination of solvents and other external agents have been proposed to achieve these tasks.

These include:

- Ethanol (EtOH)
- Methanol (MeOH)
- Dimethyl Sulfoxide (DMSO)
- Dimethyl Formamide (DMF)
- Pluronic F-127
- Bile salts (e.g. sodium cholate)
- Stained vesicles

A few examples of ways to solubilize and store VSDs commonly used in single cell studies are given below.

Example 1: di-8-ANEPPS in DMSO/ F-127 (after Rohr and Salzberg, 1994; Bullen et al., 1997). One vial (5 mg) of di-8-ANEPPS (#D-3167; Molecular Probes, Eugene, OR) is dissolved with 625 μ l of a Pluronic F-127/ DMSO solution (25% and 75% w/w resp.) for a final concentration of 8 mg/ml or 13 mM. Aliquots of 12.5 μ l (i.e., single experiment size) are made. These aliquots are stored desiccated and protected from light at 4 °C.

Example 2: RH421 in bile salts (after Meyer et al., 1997). RH421 (#S-1108; Molecular Probes) is solubilized (20 mg/ml) in the bile salt sodium cholate (10 mM in water; Sigma, C1254) at a molar ratio of about 2 to 1 to produce a 300–400x stock solution

Solubility and storage of VSDs

that can be added directly to the physiological saline bathing cells. Staining times of 3–5 minutes are normally sufficient to produce good signals. Store at 4 °C and protect from light.

Example 3: di-2-ANEPEQ in water (after Antic and Zecevic, 1995). A stock solution of di-2-ANEPEQ (also known as JPW1114; #D-6923; Molecular Probes) is made in water (3 mg/ml). Prior to microinjection this solution is filtered (0.22 µm pore size). This stock solution can be stored for several months at 4 °C.

Note: In many cases increased temperature and sonication are also required to get these indicators into solution. In general, the stock solutions of VSDs can be stored at 4°C without any loss of function or brightness.

Solubility and storage of CaSDs

The ion-sensitive indicators typically exist in two forms: free salt and acetoxymethyl (AM) esters. The requirements for solubility and storage in each case are different.

Free Salt: The salt forms of most CaSDs are water-soluble and stable for long periods at –20 °C, whether stored as a solid or in solution. Typically, these salts are used for microinjection or dialysis and therefore are prepared as concentrated stock solutions in pure (Ca-free) water. There are no special precautions required to make these solutions; however, they are easiest to deal with when made and stored as concentrated aliquots (50–100x). These aliquots are best stored desiccated at –20 °C.

Note: Some investigators mix these dyes with the internal solution of the patch pipette and store them together frozen. However, our experience indicates that dye stored in this manner will degrade faster over time.

Example 1: Oregon Green 488 BAPTA-1, Hexapotassium salt. One 500 µg vial of Oregon Green 488 BAPTA-1 (#O-6806; Molecular Probes) is dissolved in 90 µl of pure, distilled, deionized water for a stock concentration of ~5 mM. This mixture is then briefly centrifuged and sonicated to ensure complete mixing. Single experiment size aliquots are then made and stored desiccated at –20 °C.

AM ester: AM esters are normally supplied pre-aliquoted and should be reconstituted using high quality DMSO. Some AM esters also require the inclusion of a dispersing agent such as Pluronic F-127 (1–20 % w/v) to achieve complete solubility. Whether or not Pluronic F-127 is used, it is advisable to make these stock solutions at the highest possible concentration (i.e., 1–5 mM). This increases stability and minimizes the amount of solvent finally present in the bathing medium. These stock solutions should then be stored well sealed, frozen and desiccated. In fact, it is advisable to use these stock solutions immediately; otherwise the solvent will readily take up moisture, leading to decomposition of the dye.

Example 2: Calcium Orange AM for bath application. A 50 µg vial of Calcium Orange AM (#C-3015; Molecular Probes) is dissolved in a solution of DMSO/Pluronic F-127 (10% w/v) for a stock concentration of 4 mM. This mixture is then briefly centrifuged and sonicated to ensure complete mixing. The stock solution is then tightly sealed and kept on ice until used (up to 2–3 hours only).

Loading/Staining Protocols

There are a variety of potential methods for loading/staining with optical indicators. These methods fall into two main categories:

- Bulk loading
- Single cell loading

In bulk loading studies all the cells present are loaded or stained indiscriminately. Examples of bulk loading procedures include:

- Bath incubation
- AM ester loading
- Electroporation
- Cationic liposome delivery
- Hypoosmotic shock

The most popular method for introducing calcium dyes into cells is via their acetoxymethyl esters (AM). AM esters work by shielding the strongly negatively charged parts of the dye molecule (see Table 2) and hence make them membrane-permanent. Once inside the cell, nonspecific esterases cleave these esters back to their calcium-sensitive form and thus trap the dye intracellularly. In single cell studies loading is normally achieved via microinjection or dialysis through a patch pipette although localized electroporation is also an option.

We will consider the three methods most commonly used for optical recording purposes. They are:

- Bath application
- Microinjection
- Dialysis (through a patch pipette).

In each case the optimal staining/loading conditions vary depending on the indicator used. In many instances the best conditions are determined empirically; however, a few representative examples are documented here as a guide.

Example 1: Bath Application of extracellular VSDs, di-8-ANEPPS (after Bullen et al., 1997). A 12.5 μ l aliquot of di-8-ANEPPS stock solution is gently heated to melt it. To this, 1 ml of physiological Ringers is added, giving a concentration of 163 μ M. This solution is then sonicated briefly (20–30 seconds). Prior to staining, cells are washed once with PBS. Cells are then incubated at dye concentration between 75 μ M and 163 μ M. Generally ten minutes of staining is sufficient. Excess dye can be removed by a further PBS wash although is not always necessary.

Note: Avoid staining with or using VSDs in the presence of serum or large protein concentration as this can act as a sink for the dye and disrupt cell staining or even destain cells.

Example 2: Injection of Intracellular VSDs di-2-ANEPEQ into snail neurons (after Zecevic, 1996). A nearly saturated and prefiltered stock solution (3 mg/ml) of di-2-ANEPEQ is injected directly into *Helix aspera* neurons using repetitive, short pressure pulses (5–60 p.s.i., 1–50 ms) through a micropipette (resistance = 2–10 M Ω). Cells are then incubated at 15 °C for 12 hours to allow complete diffusion of the dye throughout the cell.

Example 3: Dialysis of the Intracellular VSDs, di-2-ANEPEQ, into cultured mammalian neurons (after Bullen and Saggau: Unpublished Observations). An aliquot of stock solution (5 mM) is added directly to the internal solution of the patch pipette each day for a final di-2-ANEPEQ concentration of 100–500 μ M. The internal solution of the patch pipette is (in mM): KCl 140, MgCl₂ 1, NaATP 5, NaGTP 0.25, EGTA 10, HEPES 10, pH 7.4. Seal formation and dialysis into the cell are conducted using standard methods. Diffusion of the dye away from the soma occurs at approximately 1 μ m per minute for distances less than 150 μ m.

Example 4: Bath Application of CaSDs Fluo-3 AM in cultured rat cortical neurons (after Murphy et al., 1992). Fluo-3 AM is dissolved in DMSO at 5 mg/ml and further diluted into Hank's balanced salt solution, in the presence of 0.25% pluronic F-127, for a

working concentration of 10 µg/ml. Cells are incubated with this solution for 1 hour at room temperature. These cells are then washed twice in Hank's prior to use.

Note: Avoid trying to use sharp microelectrodes for dye injection with cultured mammalian CNS neurons as this procedure has an extremely low rate of successful penetration. Dialysis through a patch pipette is a much more efficient method.

Example 5: Oregon Green 488 BAPTA-1 for dialysis via a patch pipette into cultured hippocampal neurons (after Bullen and Saggau: Unpublished Observations). An aliquot (5–15 µL) of stock solution (5 mM) is added directly to the internal solution (1 ml) of the patch pipette each day for a final Oregon Green 488 BAPTA-1 concentration of 25–75 µM. The internal solution of the patch pipette is (in mM): KCl 140, MgCl₂ 1, NaATP 5, NaGTP 0.25, HEPES 10, pH 7.4. Seal formation and dialysis into the cell are conducted using standard methods. Allow 10 to 20 minutes for the dye to equilibrate inside the cell before commencing any experimental manipulations.

Note: In experiments with calcium indicators don't include any additional calcium buffer (e.g., EGTA or BAPTA) in the internal pipette solution.

Note: Avoid using large concentrations of calcium indicator (i.e., greater than 100 µM) inside cells as this can result in significant buffering and distortion of calcium transients.

Note: When using CaSDs with patch pipettes it is important to avoid mixing of the internal solution (where [Ca] is nominally zero) and the bath saline (with millimolar calcium). For this reason it is important to apply positive pressure to the pipette when initially entering the bath. Additionally, it is wise to puff out the solution at the pipette tip immediately before seal formation.

Experimental Design Issues

There are numerous considerations critical to the design and execution of experiments with optical indicators. Many of these factors are prerequisites to obtaining useful data and avoiding artifactual results. These considerations can be divided into factors that are general to all experiments and those specific to experiments with optical indicators.

| | |
|--------------------------------|--|
| General Design Considerations | <p>Determining the authenticity of any effect arising from an experimental manipulation or drug application requires several basic criteria to be satisfied. These include:</p> <ul style="list-style-type: none"> – Measurement baseline: Was a steady baseline accomplished before an experimental manipulation or pharmacological agent was applied? – Repeatability: Was the experimental effect observed repeatable? – Reversibility: Was the experimental effect reversible upon removal of the manipulation or drug? – Graded response: Could the response be graded with stimulus strength? – Pharmacology: Can the response be blocked or potentiated with appropriate pharmacological agents? |
| Specific Design Considerations | <p>Design considerations specific to the use of optical indicators usually address aspects of dye application, signal optimization and integration with complementary electrophysiological techniques.</p> <ul style="list-style-type: none"> – Dye application: Specific criteria are needed to establish whether the indicator concentration and/or sensitivity was constant throughout the experiment. Non-uniform dye concentration or sensitivity can arise due to incomplete dialysis from a patch pi- |

ette or internalization of voltage-sensitive dye. The response to standard or a control stimulus can be used to confirm a constant responsiveness.

- Signal optimization: In some cases, the overall signal is composed of specific and nonspecific components and therefore procedures should be in place to distinguish between these components. One example of the nonspecific fluorescence is that due to cell autofluorescence. This intrinsic fluorescence can be emitted from a specimen independent of any extrinsic fluorescent molecules and is commonly a problem when illuminating biological preparation in the near UV. The solution to this problem is to measure cell fluorescence in the absence of the optical indicator and subtract this value from the resting fluorescence in the presence of the dye. This value is often measured before staining or from an equivalent unstained site. Another important experimental issue is whether or not signal averaging or digital oversampling is required to detect the signals of interest. In cases where the signals are small and averaging is required, enough similar traces must be collected to allow use of this procedure. Finally, if illumination intensity is large, consideration often needs to be given to whether a bleaching correction is required. Bleaching corrections are commonly made with control traces that are collected under experimental conditions but in the absence of the stimulus or experimental manipulation.
- Integration: The integration of optical and electrophysiological techniques often requires specific procedural changes. For instance, VSDs dissolved with solvents, especially DMSO/F-127, can inhibit seal formation between a patch pipette and the cell membrane and it is sometimes necessary to form this seal before staining the cell.

Calibration Procedures

The calibration of optical signals is necessary if the goal of an experiment is to determine a quantitative result or measure an absolute change in the parameter of interest. Likewise, if a comparison between optical signals from different experiments or between points within the same experiment is required, these signals should also be calibrated. While it may appear to be advantageous if all signals were just recorded in a quantitative manner, this approach is not always possible because other factors such as recording bandwidth are often comprised in the process. The calibration of optical signals can be achieved in one of three ways:

- Single wavelength measurements
- Ratiometric measurements
- Hybrid measurements

Without doubt, ratiometric measurements give the most reliable results. This type of measurement is possible with indicators that show a spectral shift in either their excitation or emission spectrum that is dependent on the variable of interest. These spectral shifts allow the comparison of two wavelengths where the fluorescence intensities are changing in opposite directions or between one wavelength and a spectral isosbestic point (i.e. point insensitive to the parameter of interest). In addition to providing a quantitative result, ratiometric measurements reduce or eliminate systematic variations in fluorescence due to:

- Indicator concentration
- Excitation pathlength
- Excitation intensity
- Detector efficiency.

Furthermore, ratiometric methods are important in eliminating a variety of artifacts and nonsystematic factors. These include:

- Photobleaching
- Indicator leakage over time
- Non-uniform indicator distribution
- Variable cell thickness.

In some cases ratiometric measurements are also more sensitive because the changes in fluorescence at each wavelength are usually of opposite sign and therefore the magnitude of the change in the ratio is greater than the change in either wavelength alone.

Under some experimental conditions it is impractical to perform true ratio measurements. An alternative is to perform hybrid measurements (Lev-Ram et al., 1992). In a hybrid protocol, quantitative measurements are combined with qualitative estimations performed at a different instant in time. For example, an initial baseline could be determined quantitatively with a ratiometric measurement. Subsequently, fast changes in the same parameter are followed qualitatively at a single wavelength but with much higher measurement frequency. However, it is important to note that this approach assumes all other variables (especially indicator concentration) remain constant during recording of the single wavelength measurements.

Both ratiometric and non-ratiometric methods have been used with calcium-sensitive and voltage-sensitive dyes. Examples of each type of calibration procedure are documented below. Table 6 outlines schematically how these measurements are made in each case. In addition, some general guidelines common to both types of indicator are outlined below.

VSD Calibration

Fluorescent voltage-sensitive dyes are generally considered “linear voltmeters without scale.” Specifically, they provide information about voltage changes but the absolute amplitude of this signal can vary due to differences in dye staining and variations in local sensitivity. Hence these indicators are most commonly used *uncalibrated and absolute comparisons between points and across preparations are not attempted*. However, calibrated measurements are possible in some circumstances and the success of these procedures is easily verified with concurrent electrical measurements. This type of measurement includes those made at a:

- Single wavelength
- Two excitation wavelengths based on an excitation spectral shift
- Two emission wavelengths based on an emission spectral shift

I. Single wavelength measurements for comparisons between points in same preparation: Fromherz and others (Fromherz and Vetter, 1992; Fromherz and Muller, 1994) have devised a way to compare the relative magnitude of voltage signals from different points in the same preparation. Briefly, these authors choose to examine the ratio of fluorescence changes to voltage changes of opposite sign. The rationale behind this approach is that differences in local sensitivity and the fraction of fluorescing molecules would cancel out and reflect only the ratio of the underlying voltages. Thus:

$$\frac{\Delta F_2}{\Delta F_1} = \frac{\Delta V_2}{\Delta V_1}$$

where ΔF refers to the change in fluorescence and ΔV the change in membrane potential. The subscripts 1 and 2 correspond to separate locations. Theoretically, if an electrical measurement is also made at one of these points it would be possible to calculate absolute ΔV at the other point.

Note: The sensitivity and accuracy of this approach remains to be proven and whether it is an advance over traditional data display methods (i.e., $\Delta F/F$) requires empirical evaluation.

Table 6. Calibration Methods used in Single Studies.

| Indicator | | Example | Epifluorescence configuration | Equation | Refs. |
|-----------|-------------------|-------------|-------------------------------|--|---------|
| VSDs | Single wavelength | RH421 | | $V_{m1} \approx \left(\frac{V_{m2}}{F_2} \right) \cdot (F_1)$ | 1, 2 |
| | Excitation ratio | Di-8-ANEPPS | | $V_m = CF \times R'$ | 3, 4, 5 |
| | Emission ratio | Di-8-ANEPPS | | $V_m = CF \times R'$ | 6, 7 |
| CaSDs | Single wavelength | Fluo-3 | | $[Ca^{2+}] = K_d \cdot \frac{F - F_{\min}}{F_{\max} - F}$ | 8, 9 |
| | Excitation ratio | Fura-2 | | $[Ca^{2+}] = K_d^* \cdot \frac{R - R_{\min}}{R_{\max} - R}$ | 9, 10 |
| | Emission ratio | Indo-1 | | $[Ca^{2+}] = K_d^* \cdot \frac{R - R_{\min}}{R_{\max} - R}$ | 9, 10 |

(1) Fromherz and Vetter (1992), (2) Fromherz and Müller (1994), (3) Montana et al., (1989), (4) Bedlack et al. (1994), (5) Zhang et al. (1998), (6) Beach et al. (1996) (7) Bullen and Saggau (1997), (8) Minta et al., (1989), (9) Haugland (1996), (10) Grynkiewicz et al., (1985).

II. Ratiometric methods using two excitation wavelengths and based on an excitation spectral shift for measuring absolute changes in membrane potential (after Montana et al., 1989): In addition to undergoing voltage-dependent changes in the amplitude of the emission spectrum, some VSDs also exhibit voltage-dependent spectral shifts. Loew and colleagues have used the excitation spectral shift of di-8-ANEPPS as the basis for ratiometric VSD measurements. By alternatively exciting this dye on the wings of its absorption spectrum (440 and 530 nm) and measuring wide-band fluorescence (>570 nm) they have derived a ratiometric parameter that is linear with membrane potential over the physiological range. These authors have extended this approach to include single cell imaging (Bedlack et al., 1994). By interlacing images captured at each excitation wavelength they generated a ratiometric map of membrane potential throughout a whole cell. Recently, they have extended this approach to include a more accurate calibration procedure by employing patch clamp techniques for absolute determination of membrane potential (Zhang et al, 1998). The disadvantage of this excitation ratio formation procedure is the requirement to interlace two images and/or switch excitation filters, which is time-consuming and limits the overall temporal bandwidth to less than that required to capture fast events such as action potentials.

The equation for converting normalized ratio data into an absolute membrane potential value (in mV) is:

$$V_m = CF \times R'$$

Where C.F. is the conversion factor between the ratio value and membrane potential and R' is the normalized ratio (typically normalized to R at 0 mV).

III. Ratiometric methods using two emission wavelengths and based on an emission spectral shift for measuring absolute changes in membrane potential (after Bullen and Saggau, 1999; Beach et al., 1996): An alternative method to make ratiometric determinations of membrane potential with this kind of indicator employs a single excitation wavelength and undertakes simultaneous measurements at dual emission wavelengths. This method is based on the voltage-dependent shift in the emission spectra of the voltage-sensitive dye, di-8-ANEPPS. Typically, fluorescence measurements are made at two emission wavelengths using a secondary dichroic beamsplitter (e.g., DCLP570) or prism and dual photodetectors (<570 and >570 nm). The signal at each wavelength changes in opposite directions and the ratio of these signals is linearly related to membrane potential. One implementation of this scheme employs a high-speed, random-access, laser-scanning microscope (Bullen et al., 1997) with dual photodetectors for simultaneous detection at two emission wavelengths (Bullen and Saggau, 1999). In this approach, measurements are made with a discrete laser line (476 or 488 nm) and hence acquisition speed can be very high because there is no requirement to switch excitation filters. Furthermore, because this excitation wavelength coincides with a voltage-insensitive point in the excitation spectra, the excitation spectral shift is removed as a confounding influence. Concurrent current clamp measurements can be used to calibrate this method. The formula to convert ratio values into absolute membrane potentials is the same as described in the previous section.

- CaSD Calibration Three forms of calibrated measurements are possible with this type of indicator. They are:
- Single wavelength
 - Ratiometric: based on an excitation shift or an emission shift
 - Hybrid measurements

I. Single wavelength measurements: The calibration equation for a single wavelength can be written in terms of the fluorescence values:

$$[Ca^{2+}] = K_d \cdot \frac{F - F_{min}}{F_{max} - F}$$

where

K_d = dissociation constant determined *in vitro*.

F = measured fluorescence.

F_{max} = maximal fluorescence intensity in saturating calcium.

F_{min} = minimum fluorescence intensity in zero calcium or saturation with a quenching agent (e.g., Mn^{2+}).

This kind of measurement can be undertaken with any kind of calcium indicator (e.g., Calcium Green) but is susceptible to variations in path length, dye concentration etc.

II. Ratiometric measurements When indicators are employed that shift their fluorescence spectra upon binding to calcium (e.g., the excitation spectrum of Fura-2 or the emission spectrum of Indo-1), measurements are commonly made at two distinct wavelengths (λ_1, λ_2) to obtain a ratio ($R = F_{\lambda_1}/F_{\lambda_2}$). The calibration equation for dual wavelength indicators is:

$$[Ca^{2+}] = K_d^* \cdot \frac{R - R_{min}}{R_{max} - R}$$

where

K_d^* = $K_d (F_{max}/F_{min})$,

R_{min} = ratio in zero calcium or following saturation with a quenching agent (e.g., Mn^{2+}).

R_{max} = ratio in a saturating concentration of calcium.

R_{min} and R_{max} are most accurate when they are obtained under conditions that approximate the experimental milieu (i.e., in the cell). This kind of approach is considered much more accurate than those described for a single wavelength measurement (see previous section) and overcomes discrepancies in path length, dye concentration etc.

III. Hybrid measurements: An alternative to ratiometric measurement is to perform hybrid measurements. In this case, the *resting calcium concentration* is first determined with a ratiometric measurement. Subsequently, *fast changes in the calcium concentration*, $\Delta[Ca^{2+}]$, are optically followed at a single wavelength but with much higher measurement frequency. The calibration equation for this kind of measurement (Lev-Ram et al., 1992) is:

$$\Delta[Ca^{2+}] = \left(K_d + [Ca^{2+}] \right) \cdot \frac{\frac{\Delta F}{F}}{\frac{\Delta F_{max}}{F}}$$

where $\Delta F/F$ is the fractional change in fluorescence and $\Delta F_{max}/F = (F_{max} - F)/F$ is the maximal fractional change from resting to saturating calcium concentrations and $[Ca^{2+}]$ is the resting calcium concentration measured ratiometrically at an earlier time. This kind of approach could, for example, employ Fura-2 as both a ratiometric indicator and a single wavelength dye. Normally this indicator requires mechanical filter switching, which is an inherently slow process, to alternate excitation wavelengths. However, by alternately employing dual and single wavelength measurements the hybrid approach overcomes this limitation and allows faster estimations of calcium changes. It is

important to note that this approach requires an invariant indicator concentration during recording (i.e., no bleaching or change in dye concentration from extrusion or dialysis).

General guidelines for the calibration of optical indicators

An important step in converting optical measurements to the physiological parameter of interest is the *post-experiment calibration*. While the conversion factors for both voltage and calcium indicator calibration can be determined in solution or various simplified preparations (i.e., vesicles), these conditions often do not approximate the true intracellular milieu. Factors that are not well reproduced in these situations include:

- Temperature
- pH
- Ionic strength
- Interaction of dyes with proteins or membranes.

Moreover, the interactions of some CaSD with intracellular proteins have been shown to change the apparent K_d (Kurebayashi et al., 1993). In short, in situ calibrations are typically better than equivalent in vitro procedures and should be adopted whenever possible.

These calibrations are typically achieved by chemical clamping of the cell with pore-forming ionophores. For example, ratios of membrane potential can be calibrated with valinomycin. Specifically, a series of valinomycin-mediated K^+ diffusion potentials are used to step through the range of interesting membrane potential while measuring fluorescence ratios. For a detailed description of this procedure see Loew (1994). Likewise, ratiometric calcium measurements can be calibrated in situ with the ionophores such as ionomycin or calcimycin (or its analog 4-bromo-A-23187). However, care should be taken when using these compounds because they possess a relatively high level of autofluorescence, especially in the UV. For a detailed description of these procedures see the chapter by Kao (in Nuccitelli, 1994). This chapter also documents many of the underlying assumptions and practical limitations of these calibration procedures.

Note: Always subtract off any autofluorescence or other offsets before forming a ratio or calculating $\Delta F/F$.

Signal Processing Methods

Even after the brightest indicators have been chosen and instrumentation considerations optimized, some optical signals are weak and/or noisy. In other instances, the sensitivity of the probes being used can be poor or the underlying physiological events are quantal in nature. In each case, extra care must be taken to extract the signal from background noise. Noise can be:

- Systematic
- Random

In some cases systematic noise can be measured and removed by subtraction or division. In contrast, random noise is harder to separate from the underlying signal. Signal averaging is one way to cancel out the effects of truly random noise. However, averaging is not always possible (i.e., non-stationary events). Within a single sweep, only those components of random noise that are spectrally separate from the signal can be removed (i.e., by filtering).

To overcome noise problems in optical recording experiments a number of signal processing and noise reduction techniques are available. These include:

- Source noise ratio formation
- Digital filtering
- Signal averaging

Systematic noise present in experimental records can be of two types: additive or multiplicative. Typically, additive noise can be removed by subtraction while multiplicative noise is best corrected by ratio formation. Multiplicative noise generated by variations in source intensity is the most common noise source in optical recording experiments. This is particularly apparent in cases where the relative changes in fluorescence are equal to or less than the fluctuations arising from the light source. In this case, it is difficult to resolve the signal from the noise. This is a particularly prevalent problem with laser sources where such intensity variations can be as much as 5 % peak-to-peak. However, these variations can be measured and removed from the signal by ratio formation. In practice, ratio formation between the signal and a reference measurement is the most efficient way to remove source noise variations. The advantage of this scheme over subtraction is that there is no requirement to match amplitudes between signal and reference. The effectiveness of this procedure is documented in Bullen et al. (1997) and is demonstrated with real signals in Figure 5.

Source noise ratio
formation

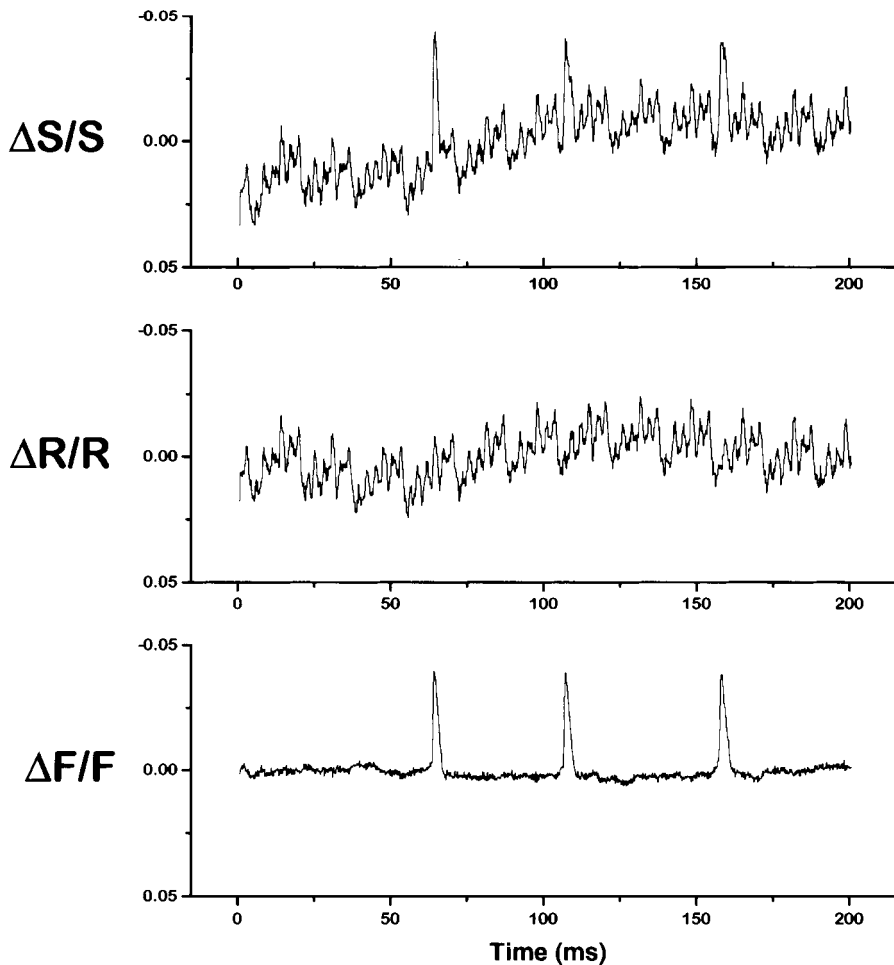


Fig. 5. Demonstration of Source Noise Removal following Ratio Formation with a Reference Signal. Top trace ($\Delta S/S$) is the raw signal that includes both the underlying signal and the contaminant source noise. Middle trace ($\Delta R/R$) is a reference signal containing the source noise sampled directly from the excitation source which in this case was a laser. The bottom trace ($\Delta F/F$) is the processed signal after source noise has been removed. Note how the source noise from laser intensity fluctuations is completely removed by this procedure.

Another way to remove source noise variations is to form ratios between two emission wavelengths. In this case the source noise variations are present in both wavelengths as common mode signals and effectively cancel out in the ratio formation process.

Digital Filtering Digital filtering is an important signal conditioning tool used to reduce the contribution of random noise or unwanted signals (cf. Chapter 45). This is especially true for non-stationary signals or for signals that cannot be averaged. The principle behind digital filtering is that frequencies of interest can be separated based on whether they represent signal or noise. There are four main types of digital filters:

- Low-pass
- High-pass
- Band-pass
- Band-stop

Low-pass filters are important in restricting the bandwidth of experimental records to that containing useful signal components while removing high-frequency components. *High-pass* filtering is also referred to as *A/C coupling* and is useful in removing the DC component of a signal and thus highlighting only that part that is changing. *Band-stop* (or *notch*) filters reject discrete frequency bands and are particularly useful in removing AC-line noise from experimental records.

Other special filters exist that conserve high frequency components while still acting as a *low-pass* filter. One example is the Savitzky-Golay filter method which essentially performs a local polynomial regression to determine the smoothed value for each data point. This method is superior to other filtering methods because it tends to preserve features of the data such as peak height and width, which are usually 'washed out' by adjacent averaging or *low-pass* filtering.

Different implementations of these digital filters are commonly found in scientific plotting and graphing packages (such as Origin or SigmaPlot) or in specialized mathematical environments (such as Matlab or Mathematica).

Note: Care must be taken to avoid filtering frequencies that contain important signal components. Moreover, it should be recognized that some filtering methods can introduce small phase shifts into the data. However, modern finite impulse response (FIR) digital filters can be used in opposite directions to counteract this problem. Finally, care should be taken to ensure the sampling theorem is not violated (see "Data Acquisition and Digitization Issues").

Note: It is advisable to use analog filters (active or passive) at all data acquisition and processing steps (i.e., from the detector onwards). This typically reduces the accumulation of unwanted noise at each step and reduces the need for subsequent digital filtering.

Signal Averaging Signal averaging refers to the grouping in space or time of areas or repeated trials to reduce the contribution of random noise (cf. Chapter 45). If the noise is truly random, then signal averaging will reduce it by a factor of $1/\sqrt{N}$, where N is the number of trials. This kind of averaging requires stationary events and involves no loss in frequency components occurring, provided the signals being averaged are strongly time-locked. If signal averaging procedures are adopted, care should be taken not to introduce any temporal jitter (i.e., biological or instrumental) into the process as this can result in a low-pass filter effect. In cases where this is a problem, events such as action potentials can be aligned by their peaks and averaged together to improve the overall signal quality.

The effectiveness of signal averaging is demonstrated in Figure 6. In this example, drawn from experiments examining the linearity of ratiometric VSD data, the relative noise decreases in direct proportion to the number of trials averaged.

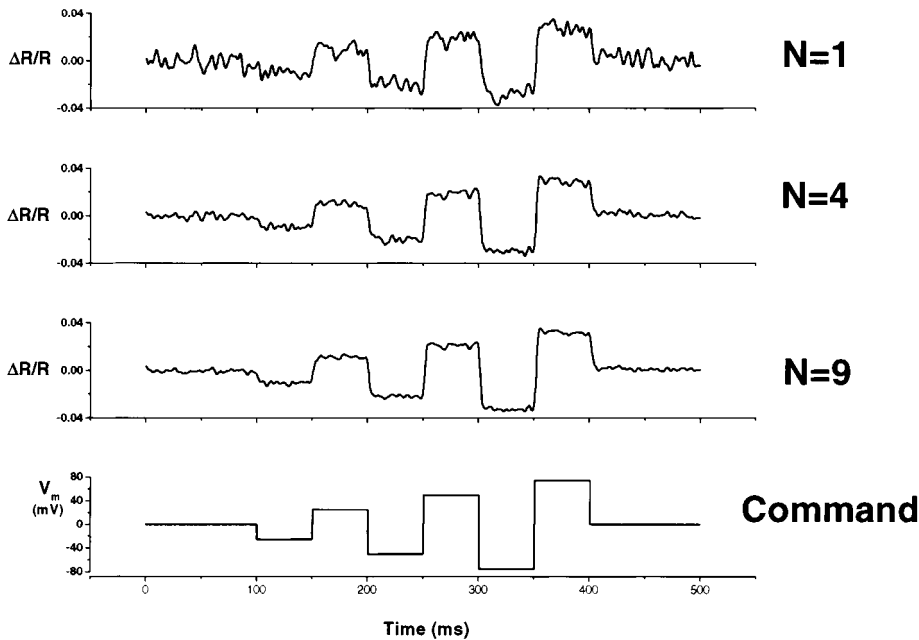


Fig. 6. Example of Noise Reduction by Signal Averaging. The top three traces represent ongoing average of ratiometric signals recorded from the same scanning point under identical conditions during an optical calibration experiment. The number of traces averaged is given to the right of each trace. The bottom trace is the voltage command waveform used to elicit these signals. Note the degree of improvement (i.e., the reduction in noise) with the increasing number of traces averaged. Experiment conducted with the voltage-sensitive dye di-8-ANEPPS in cultured hippocampal neurons.

One disadvantage with temporal averaging is that the overall measurement frequency is typically reduced because of the time required to capture enough traces for averaging. This restricts the number of points that can be sampled within a fixed time frame. This can be a problem in cases where the response time course to a drug or an experimental manipulation is important.

■ Results

This section documents a number of considerations important in the presentation and display of experimental results. These include:

- Data presentation
- Important properties of experimental records
- Types of experiments and representative records

Data Presentation

The results of optical recordings from single neurons or parts of neurons can be presented in a number of ways. These include:

- One-dimensional records: Signals recorded from a single site or data extracted from individual points or regions of interest (ROI) in an image and displayed as a one-dimensional trace against time.

- Pseudo-color images: A series of images colored in a way to distinguish activity levels or changes in ion concentration.
- Live video: A reproduction, sometimes with altered timing, derived directly from the images captured during the experiment.

While video records and pseudo-color images provide a graphically pleasing and qualitative means of displaying data, it is often difficult to show time courses and/or quantitative changes with this format. Furthermore, it is often difficult to reconcile these images with other parameters measured concurrently (i.e., currents and voltages) that are inherently one-dimensional.

Important Properties of Experimental Records

An unfortunate trend in modern papers is presentation of heavily reduced data and in many cases original records are omitted completely. This is especially true in studies employing optical recording techniques where many recordings are often reduced to a single pseudo-color image. However, for others to judge the quality of the underlying data it is important to present examples of original records. In examining this kind of data it is important to consider a number of different questions. For instance, do the records presented show:

- Detection sensitivity: Were the recording method and indicator(s) used sensitive enough to make useful measurements?
- Signal-to-noise ratio: Is the signal-to-noise ratio exhibited sufficient to make experimentally useful conclusions and could it be further optimized?
- Spatiotemporal resolution: Did the method used possess sufficient spatiotemporal resolution to answer the experimental questions posed?
- Fidelity: Are the records presented an accurate reflection of the underlying physiological events or was some disruption or alteration caused by the recording methodology itself?

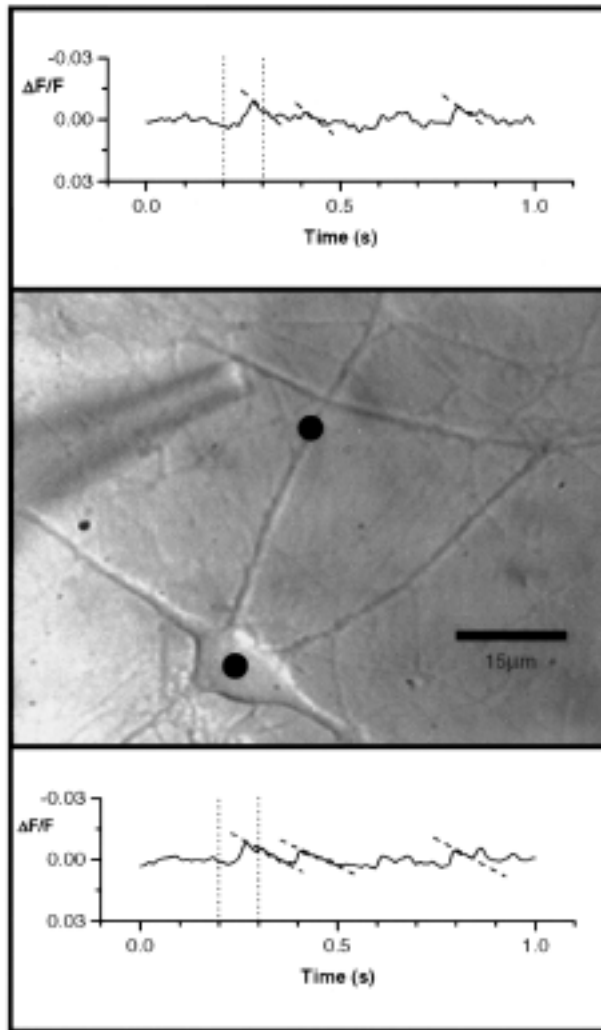
Types of Experiments and Representative Records

A number of experimental records are presented here to demonstrate the types of applications possible in experiments with single neurons. For instance, the ratiometric signals presented earlier (see Figure 6) were derived from an experiment examining the linearity of the VSD di-8-ANEPPS. This record was made from a scanning site immediately adjacent to a patch pipette and demonstrates the strong similarity in terms of response time and amplitude between the optical signals and voltage-clamp command waveform. This type of calibration can subsequently be used to quantify similar optical measurements made under more physiological conditions.

In a separate example using the same VSD, the pattern of postsynaptic potential integration and conduction in the dendrites of cultured hippocampal neurons were examined (see Figure 7.). Several facets of this experiment further illustrate the usefulness of this kind of optical recording. In particular, these recordings were made from very small cellular structures in a noninvasive manner. Moreover, several measurements were made simultaneously from different recording sites (2 μm diameter) and in way not easily possible with other experimental methods. Furthermore, these signals were obtained at rates (i.e., 2 kHz) sufficient to adequately sample the physiological events of interest.

In a similar experiment, the spatial differences in calcium signals generated from adjacent points in the same cell following a series of action potentials were examined

Fig. 7. Representative Voltage-sensitive dye recordings. Optical recordings of postsynaptic potentials in the dendrites of hippocampal neurons made with di-8-ANEPPS at two independent scanning sites. Focal stimulation of presynaptic terminals undertaken with hyperosmotic saline occurs between the two dashed lines.



(Figure 8.). This experiment demonstrates spatial differences in the magnitude and kinetics of calcium transients examined at different points within the same cell at high temporal resolution.

■ Troubleshoot

This section documents a number of problems commonly encountered in optical recording experiments. In particular the following issues are examined:

- Signal quality issues
- Prevention of photodynamic damage
- Loading and staining problems

Signal Quality Issues

The most important factor in all recording techniques is the magnitude of *signal-to-noise ratio* (S/N) that can be achieved. This is especially true for some optical indicators (especially VSDs) where the relative change in fluorescence can be very small. Many factors can influence signal quality. These include:

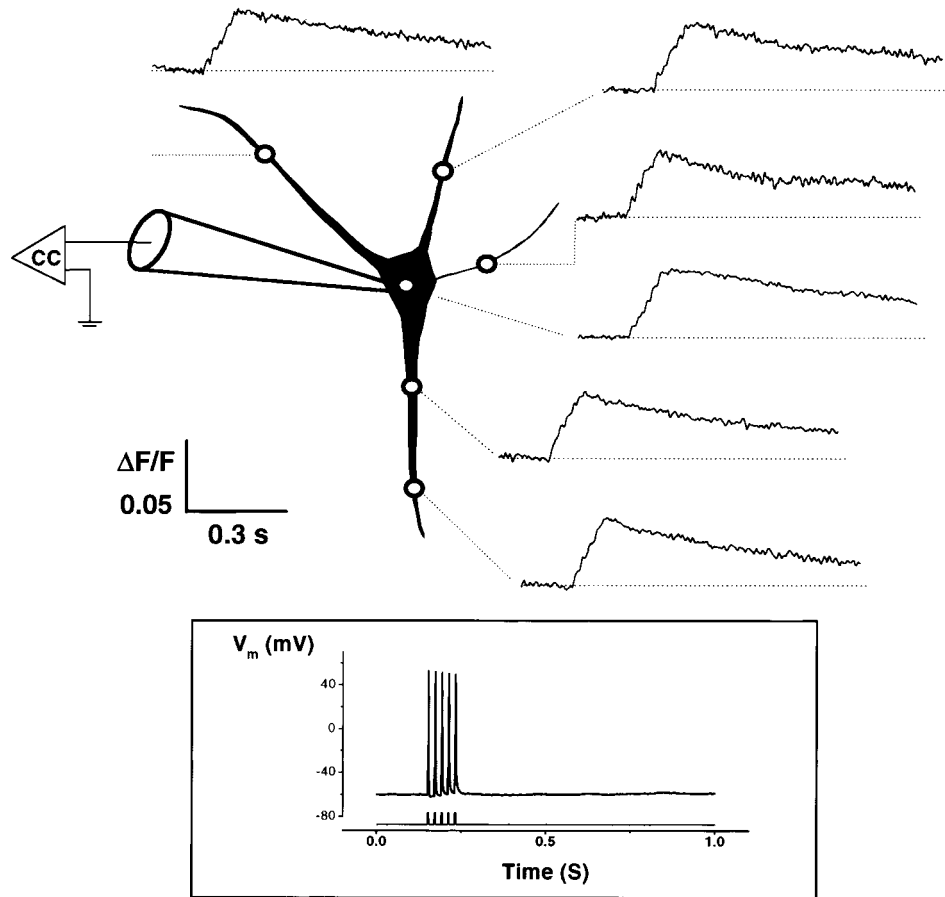


Fig. 8. Representative Calcium-sensitive dye recordings. Optical recordings of a calcium transient elicited by somatic current injection (see inset) in a hippocampal neuron and measured with Oregon Green BAPTA-1 (100 μ M). Measurements made with a Random-access, laser-scanning microscope (after Bullen et al., 1997). The relative position of each scanning site (2 μ m) is indicated on an outline drawing of the cell.

- Excitation intensity: The fluorescence arising from an optical indicator is directly proportional to the excitation intensity and hence the strongest light source possible should be employed. However, care should be taken to avoid bleaching and phototoxicity associated with excessive illumination intensity.
- Indicator concentration: Emission light intensity is also proportional to the indicator concentration and hence the maximal amount of probe possible should be utilized. In this case, care should be exercised to avoid probe concentrations that have pharmacological or buffering effects and thereby change the underlying physiology (see section "Loading and Staining Problems"). Moreover, at very high indicator concentrations emission light intensity begins to decline due to "quenching."
- Excitation volume: In scanning microscopy, the signal from a large excitation volume (i.e., large scanning spot) will be greater than from a smaller one because more fluorophores are excited. Hence there is a tradeoff between the spatial resolution achieved with a small scanning spot and the consequent reduction in excitation volume and signal strength.
- Indicator sensitivity: The best signal quality is likely to arise from optical indicators with large sensitivities (i.e., large $\Delta F/F$, F_{Ca}/F_{Free} or R_{max}/R_{min}) and therefore in cases

where there is a choice between indicators, the candidate with the largest sensitivity should be preferred.

- Indicator brightness: When a choice between indicators exists it is always advisable to choose the brightest one. Guidelines on comparing absolute indicator brightness are included in a later section (“Criteria for Comparing Indicators”).
- Maximum collection efficiency (N.A. and Filters): It is important to remember that collection efficiency is determined by the product of many factors. These include the numerical aperture, the absolute transmission of the objective lens, the relative throughput of the microscope and the transmission of the dichroic mirror and related filters. A total collection efficiency of 10% would not be uncommon and in a worst case scenario this value could be much lower. Thus, in cases where there is a choice between objective lenses it is always best to choose the lens with the highest N.A.. This serves to maximize the illumination intensity and to optimize the collection of emission light. Similarly, care should be taken in the selection of dichroic mirror and emission filters such that large portions of the signal are not excluded. Other potential sources of light loss include: the use of DIC optics without the analyzer removed from the emission pathway, dirty optics or optical misalignment. Just the DIC analyzer alone will reduce emission light intensity by another 50%.
- Maximum detection efficiency (Q.E.): In all cases, but especially when light levels are low, it is advantageous to choose a detector with the highest possible quantum efficiency. This ensures that all photons captured are converted into a useful signal (see Table 5).
- Recording bandwidth: Recording bandwidth should be just enough to capture signals of interest. Inclusion of temporal bandwidth in excess of that needed to record the signal will only contribute noise. However, if extra temporal bandwidth exists, it can be used to improve signal quality by *digital oversampling*. Oversampling is a form of temporal smoothing that averages several closely spaced points where only one was previously sampled. This procedure serves to reduce random noise with the improvement in the signal-to-noise being proportional to $1/\sqrt{N}$, where N is the number of extra samples.

Signal Quality Checklist

- ✓ Maximum-tolerated Excitation Intensity
- ✓ Maximum Probe Concentration
- ✓ Most Sensitive Indicator
- ✓ Brightest Indicator available
- ✓ Maximum Collection Efficiency (N. A. & Filters)
- ✓ Maximum Detection Efficiency (Q. E.)
- ✓ Minimum Bandwidth (or max. oversampling)

Prevention of Photodynamic Damage

Excessive illumination intensity can sometimes lead to photodamage or phototoxicity. In many cases this phenomenon arises from the production of free radicals, such as singlet oxygen, which are a byproduct of fluorescent excitation. Among other things, these free radicals can severely disrupt membrane integrity. This is a particularly prevalent problem for VSDs that reside in the plasma membrane. There are various methods for reducing photodamage from fluorescent indicators. Some of these methods attempt to

Table 7. Antioxidant Formulations for Use with Fluorescent Indicators.

| | Mol. Wt. | Solubility | Max. Soluble concentration | Best Storage Option | Stock Concentration (1, 5) | Final Concentration |
|------------------------------------|----------|------------|----------------------------|---------------------|----------------------------|---------------------|
| Ascorbic Acid (A4403) (2) | 176.1 | Water | 10 mg/ml | -20°C | 11 mM or 2 mg/ml | 110µM |
| Citric Acid (C5920) (2) | 294.1 | Water | 100 mg/ml | Room Temp or -20°C | 27mM or 8 mg/ml | 270µM |
| Glutathione (3) (G6013) (2) | 307.3 | Water | 50 mg/ml | Use Fresh | Use Fresh | 32µM or 1 mg/100 ml |
| Pyruvate (P5280) (2) | 110 | Water | 100 mg/ml | -20°C | 25mM or 2.8 mg/ml | 250µM |
| Tocopherol (4) (T1539) (2) | 430.7 | 100 % EtOH | 25 mg/ml | -20°C | 46mM or 20 mg/ml | 46µM |
| Tocopherol Acetate (4) (T1157) (2) | 472.8 | 100 % EtOH | 25 mg/ml | -20°C | 42mM or 20 mg/ml | 42µM |

Notes: (1) Make fresh each day as some components are not stable more than 3 hours. (2) Sigma Catalog Numbers included for each reagent. (3) Reduced form of Glutathione. (4) May also require warming to get into solution. (5) Either 100x stock in physiological saline or 1000x stock in ethanol.

minimize photodamage by the addition of large quantities of antioxidant to the bathing solution, others employ a lesser amount of strategically placed antioxidant (i.e., in membranes) while still others act by removing oxygen from the solution altogether. Some examples of these approaches are:

- ACE et al.: The simplest strategy to reduce the effect(s) of free radicals in solution is to add large quantities of antioxidant to the bathing medium. Various formulations of vitamins (A, C and E; ACE) and other similar agents (e.g., Trolox; Fluka) have been suggested. However, the reported success of these formulations is relatively modest, probably due to the lack of immediate protection at the site of injury (i.e., membrane). Representative agents of this sort, together with solubility and storage information, are documented in Table 7.
- Astaxanthin: A more direct approach employs the natural carotenoid, Astaxanthin. Initially this agent was utilized as an antioxidant for use in combination with a novel VSD based on fluorescence-resonance energy transfer principles (Gonzalez and T sien, 1997). The effectiveness of this approach should in principle be greater than antioxidants in solution because of its physical proximity in the membrane and its potent ability to quench reactive oxygen species. Furthermore it has been shown that relatively large concentrations of this agent can be used without fear of toxic effects. This, together with its relative water solubility when compared to other carotenoids such as β -carotene, make it an extremely attractive antioxidant agent. However, preliminary reports describing the use of Astaxanthin with other VSDs have revealed only modest effectiveness. For information about the solubility, storage and application of Astaxanthin, see Cooney et al. (1993).
- Glucose Oxidase/Catalase: The combination of glucose oxidase and catalase is a potent oxygen removal scheme and is particularly effective when used with the VSD di-8-ANEPPS (Obaid and Salzberg, 1997). Glucose oxidase takes oxygen from solution to form H_2O_2 which is subsequently converted to water by catalase. The high catalytic activity of these enzymes means that relatively small amounts (G.O. 40U/ml and Cat. 800U/ml) are needed to de-oxygenate a solution. Glucose Oxidase (G-6125) and Catalase (C-40) are both available from Sigma (St. Louis, MO).
- Oxyrase: Oxyrase is a biocatalytic oxygen reducing system. This commercial preparation also works by removing oxygen from the bathing solution surrounding cells. Oxyrase is prepared from *E. coli* and is a crude preparation that uses lactic acid, formic acid or succinic acid added to the bathing solution as a hydrogen donor. Prelim-

inary experiments with this formulation and fluorescent dyes indicate it is an effective antioxidant in many instances. Oxyrase is available from Oxyrase Inc. (Mansfield, OH).

There is no universal solution to the problem of photodamage. In many cases the best method for a particular application must be determined empirically. However, in general terms some of the above methods are more effective than others. In our experience with VSDs the Glucose Oxidase/Catalase combination is the best method for use with single cells in short-term experiments.

Comparison of antioxidant formulations

Loading and Staining Problems

There are three types of problems commonly associated with VSD staining. They are:

VSDs

- Poor staining/Low membrane affinity: Some VSDs (e.g., RH414) have a relatively modest membrane binding affinity for some cell types while others stain cells at a particularly slow rate (e.g., di-8-ANEPPS). The binding affinity of these dyes is due in part to their structure but membrane composition may also play a role. Furthermore, there are significant species and cell type differences (Ross and Reichardt, 1979). There are three potential solutions to this problem. Firstly, a series of suitable dyes should be screened prior to beginning a new series of experiments to find those which exhibit the highest membrane affinity and largest signal amplitude. Secondly, a wide range of staining time and conditions should be considered to determine the optimal staining conditions. Finally, there are agents (i.e., 0.05% Pluronic F-127) that can be added to the dye stock solution to facilitate dye insertion into the membrane.
- Overstaining: Using excessive amounts of VSDs can have various deleterious effects. In particular, nonspecific fluorescence arising from unbound dye or dye bound in a nonspecific orientation can seriously degrade signal size and quality. Furthermore, some VSDs have been shown to exhibit pharmacological effects when used at high concentrations and thus it is recommended that the minimal dye concentration compatible with good signals should be employed.
- Dye internalization: Because VSDs reside in the outer leaflet of the plasma membrane there is always the possibility that some dye molecules can cross into the inner leaflet or even directly onto membranes of intracellular organelles. Various membrane recycling processes can also serve to transport VSDs into cells. Such internalization of VSDs typically results in decreased signal size because these dye molecules have either no sensitivity to membrane potential or a directly opposite sensitivity to that of normally oriented dye molecules. The solution to this problem is to use dyes that are less likely to be internalized (e.g. di-8-ANEPPS). Additionally, incubation at greater than room temperature apparently increases the likelihood of dye being internalized into the cell, so this should be avoided.

There are four types of problems commonly associated with loading cells with CaSDs. Two of these problems are specific to AM ester loading techniques. They are:

CaSDs

- Compartmentalization: Under ideal conditions, fluorescent indicators loaded with this technique are uniformly distributed in the cytosol and absent from other cellular compartments. However, AM esters are capable of accumulating in any membrane-enclosed compartment within the cell. In addition, some polyanionic forms of these indicators can be sequestered within various organelles by active transport processes. This aberrant compartmentalization is normally more pronounced at higher loading temperatures and can be avoided by reducing loading temperature. The use of indicators conjugated to dextran can also retard compartmentalization and sequestration.

- Incomplete ester hydrolysis: Low or slow rates of de-esterification can result in a significant proportion of intracellular dye being partially de-esterified and hence insensitive to calcium but still somewhat fluorescent. This can result in a serious underestimation of the true cytosolic calcium concentration. Additionally, incomplete ester hydrolysis can also promote compartmentalization. Fluorescence quenching by Mn^{2+} , which only binds to the de-esterified form, is one way to quantify this effect. One means to avoid the confounding effects of fluorescence from AM esters is to select indicators whose esters are non-fluorescent. For instance, Calcium Green and Oregon Green 488 BAPTA are basically non-fluorescent as AM esters. In contrast, AM esters of Fura-2 and Calcium Orange still possess some basal fluorescence.

Two additional problems occur with both AM ester loading and micro-injection or dialysis of free salt forms of CaSDs. They are:

- Overstaining: Whether AM esters or simple salts are used to load single neurons, some care must be taken to not overload the cells as this can cause unwanted buffering effects. This buffering can affect the resting calcium concentration, the size and kinetics of calcium transients and disrupt various cellular processes dependent on calcium. In short, the consequences of using incorrect indicator concentrations vary from poor signal quality to distorted physiology. One way to demonstrate that the signals recorded are free of any buffering effects is to conduct experiments across a range of indicator concentrations.
- Extrusion: Various anionic indicators tend to leak out or are actively extruded by some cell types. In some cases various pharmacological tools can be used to block this problem (i.e., probenecid, sulfinpyrazone and verapamil). Another solution is to use indicators designed to be resistant to these phenomena. In particular, dextran-conjugated dyes are typically resistant to extrusion and leakage. Recently, Texas Fluorescence Labs (Austin, TX) have developed a number of such calcium dye variants that are leak-resistant (i.e., Fura PE3, Indo PE3 and Fluo LR).

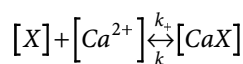
■ Comments

This section examines various issues of secondary importance that should be considered in the design of optical recording experiments. These include:

- Indicator binding kinetics
- Criteria for comparing indicators
- Data acquisition and digitization issues

Indicator Binding Kinetics

Ion-sensitive indicators bind to free ions and form a complex in a process described by simple bimolecular binding kinetics. Binding of free indicator $[X]$ and free calcium $[Ca^{2+}]$, and unbinding of bound calcium $[CaX]$ are determined by the *on-rate* and *off-rate* (k_+ and k_- , respectively).



There are several important consequences that arise from this simple binding scheme. Firstly, these indicators exhibit a classical sigmoidal-binding curve and thus the relationship between calcium concentration and fluorescence is not linear. However, a straight line can be used to approximate this relationship over the steepest part of the

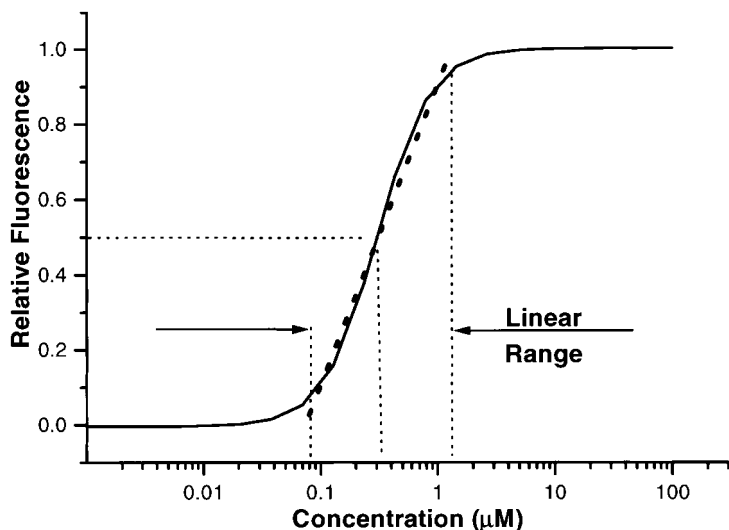


Fig. 9. Typical Binding Curve for a Calcium-sensitive dye. Binding curve of an idealized calcium indicator with a $K_d = 350$ nM. Note how the linear range of this indicator (dashed line) extends from about 0.1 x to 10x the K_d .

curve (see Figure 9). An important consequence of this simplifying assumption is that the linear range of such an indicator only encompasses calcium concentrations between 0.1 x and 10x the K_d (see the example in Figure 9). Outside this range, the fluorescence produced is not related to the underlying calcium concentration in the same linear fashion. To overcome this limitation each kind of calcium indicator is commonly available in a range of affinities (see Table 2). In choosing between calcium indicators of different affinities, it is safest to match high end of this linear range with the maximum concentration expected.

Another limitation arising from the kinetics of these indicators is the relatively slow off-rate, especially in high-affinity versions of these molecules. A functional consequence of this rate is that the decline in fluorescence measured during a typical calcium transient probably reflects the rate of dye unbinding rather than the rate of calcium removal. Furthermore, it is unlikely that fast repetitive events will be adequately tracked with these probes.

Another important consideration in experiments examining the diffusion of calcium within cells is that the Ca-dye complex can diffuse with faster kinetics than calcium alone, which is subject to cytosolic buffering. Thus, it is possible to get the incorrect impression of actual calcium movement.

Note: The use of dextran-conjugated dyes significantly decreases the magnitude of dye diffusion and reduces the likelihood of artifactual calcium movement.

Criteria for Comparing Indicators

The extensive array of available indicators often requires the investigator to choose between many seemingly similar dyes. Objective criteria for comparing different dyes typically includes consideration of their:

- Sensitivity
- Brightness
- Photostability

- Sensitivity** The sensitivity of an optical indicator refers to the magnitude of its change in fluorescence relative to the change in the parameter it is being used to measure. Typically, a sensitive indicator will undergo a large change in fluorescence for even a small change in ion concentration or voltage. However, while some dyes (e.g., Fluo-3) exhibit very large sensitivities, they are not necessarily suitable for all applications because they lack brightness.
- Brightness** Brightness refers to the strength of the fluorescence generated by an optical indicator. The fluorescence output from a given dye depends on the efficiency with which it absorbs and emits photons and its ability to undergo repeated excitation/emission cycles. Absorption efficiency is normally quantified using the molar extinction coefficient (ϵ) which is a value typically determined at the peak of the excitation spectrum. Fluorescence is normally characterized by its quantum efficiency (QE), or the ratio of photons emitted per photons absorbed. The quantum efficiency is normally a measure of total emission over the entire emission spectrum. Brightness or fluorescent intensity per dye molecule is proportional to the product of ϵ and QE and is the most useful way to compare the potential signal of two similar indicators.
- Photostability** Under intense illumination, the irreversible destruction or photobleaching of particular fluorophores becomes a limiting factor in some experiments. While there is little objective information to compare the photostability of different indicators, some recently developed fluorophores (i.e., Oregon Green) have been engineered for improved photostability over their predecessors (i.e., Fluorescein).

Data Acquisition and Digitization Issues

In order to process and/or store analog optical signals in a computer, they have to be digitized (cf. Chapter 45). The interface between the analog and digital world is commonly a device called an analog-to-digital converter (A/D converter). In general, the selection of an A/D converter depends on two parameters:

- Digitizing resolution
- Speed of conversion

- Resolution** The functional digitizing resolution required in an experiment depends on the relative intensity change in the signal. For instance, a typical VSD signal ($\Delta F/F$) is 1 % per 100 mV and an experimentally useful resolution would be 2 mV in membrane potential (i.e., 1/50 of 1%). The corresponding digitizing resolution needed under these conditions would be 5000 digitizing steps ($50 \times 100 = 5000$). This number corresponds to 13 bits of digitizing resolution (i.e., 8192 steps) because 12 bit digitization (i.e., 4096) exhibits insufficient sensitivity. Failure to employ sufficient digitizing resolution will lead to the appearance of digitizing noise (i.e., quantized steps) in experimental records and/or the failure to adequately resolve the signals of interest.
- Speed of Conversion** The speed of an A/D converter should be high enough to adequately reproduce all interesting frequency components in the signal. The sampling rate (f_{sample}) of an A/D converter depends on the *temporal bandwidth* of the signals (Δf) and the number (N) of the channels sampled simultaneously:
- According to the sampling theorem this is the minimal sampling rate that can adequately reproduce the signal. Ideally, an oversampling factor of 2–5 should be employed. Typically, the frequency bandwidth for electrophysiological signals is between 500 Hz and 5 kHz and thus the minimal f_{sample} is 10,000 times the number of channels sampled

in parallel. This factor becomes a serious consideration when a large number of points or channels are recorded and digitized serially (e.g., an image). Moreover, maximum digitization rates typically scale inversely with digitizing resolution. Thus, under circumstances where signal sizes are small and 14- or 16-bit digitization is required, the throughput of an A/D converter can severely limit the bandwidth of optical recordings.

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