Chapter 12

Intracellular ion measurement with fluorescent indicators

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1. Introduction

Most of this book deals with electrical recording techniques which, although powerful, cannot always be used to study the composition of the intracellular environment - particularly when small cells or populations of cells are the preparation of interest. Optical techniques are becoming an increasingly attractive alternative method because of their *apparent* non-invasive nature and ease of use. One area in which optical techniques have largely replaced other methods is the measurement of intracellular ion concentrations with fluorescent indicators. This chapter will provide an introduction to this increasingly important application and will also consider some of the basic features of optical versus electrical recording techniques.

Electrical recording necessarily measures the movement of electrical charge. Individual charges can neither be created nor destroyed, and the overall balance between positive and negative charge must remain within narrow limits, since only a small charge separation causes a large electrical potential difference (voltage). Electrophysiology revolves around the measurement and/or control of charge separation and, as described elsewhere, the separation of charge by ion selective membranes allows determination of the ion levels within the cell as well as the creation of the Nernstian membrane potential. Some of the problems associated with electrical measurements arise from the fact that energy must be extracted from a system in order to ascertain its properties. Although careful electronic design can minimise the extraction of energy from the system (cell), the measurement process must perturb the system. In contrast to the idea of extracting energy from the system to measure its properties, with optical techiques (such as fluorescence and absorbance measurements) the experimenter actually supplies energy to examine the system, by supplying photons to the system and recording the results of the interaction of the photons with the system. This has several important consequences that should be kept in mind: (1) Photons will interact with any molecule that has absorbance in the

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wavelength range of the photon (thus molecules that are of no interest to the experimenter may also produce a signal that will contaminate the experimental record). (2) It is usually necessary to introduce special molecules into the cell so that specific cell functions can be examined (and the introduction of these "probes" will perturb the system). (3) The amount of material that is being examined with the photons may be small so that there will be quantal limitations in the signal to noise ratio that can be achieved. (4) Energy is being supplied to the system, which at the very least will generate heat. At the other extreme, the molecules that absorb the photons may be destroyed (cf. photolysis techniques). Put in more concrete terms for fluorescence measurements, these consequences take the form of: (1) Cell autofluorescence. (2) Difficulties associated with introducing molecules that bind the ions of interest (either by intracellular injection or allowing the molecule to diffuse into the cell). (3) The small intracellular volume and limited concentrations of indicator or ion that binds to the indicator produce "shot noise" in the experimental record (see subsequent discussion). (4) Photon-induced degradation of the indicator (bleaching) and cell damage.

Optical and electrical recording techniques share a fundamental property in that their signals are both quantised. Although for convenience we may regard electrical or optical signals as being continuous, they in fact consist of individual charges or photons, and the statistical variation in their rate of arrival constitutes a source of noise. In the case of electrical measurement, this particular form of noise is not usually significant. For example, even the very small currents that are recorded in patch-clamp experiments represent the flow of a large number of electrons; e.g. it takes the movement of about 6 million electrons per second to generate a current of only 1pA. However, the question of time resolution must also be considered, and the timescale of interest is often in the millisecond range or less. Over these intervals, only a few thousand charges will be moving, and so the quantal nature of the current becomes much more important, to the extent that it may become significant.

The quantal nature of optical signals can be observed more readily. For example, in a cell whose volume is 10 pl and resting calcium concentration is 100 nM there are about 600,000 free calcium ions. In order not to perturb the system too much a fluorescent indicator is chosen that binds to only 1% of the free ions, resulting in the need to detect about 6000 indicator molecules. Assuming that each molecule gave off 100 photons per second (at this rate the molecules would probably bleach very quickly!) and with a very good detection system with 10% efficiency, the photon arrival rate would be 60,000/s. This is two orders of magnitude lower than the flux of electrons associated with the 1pA current described above. For such low numbers of particles, statistical variation in the number detected becomes a major problem. Optical detection is a Poisson process, where the variance of the signal is equal to its mean, so that if a 10 ms time resolution is desired the best that might be obtained in this example would be a signal-to-noise ratio of about 20 (defining the signal-to-noise ratio as the mean signal divided by the standard deviation of the signal). Although at this high photon detection rate the signal-to-noise ratio is acceptable, poor optical design can easily reduce the detection efficiency to <1% and make the signal too

noisy to be useful (at a 10 ms time resolution). Actually, most calcium indicators bind much more than 1% of the free calcium ions, which has the advantage of providing larger signals, but at the expense of greater disturbance to the cell by the measurement process.

The above calculation highlights some important areas for the measurement of ions with optical methods: (1) the importance of efficient optics and detector design; (2) the choice of affinity of the indicator for the ion of interest. (Note that further amplification of the signal will not improve the measurement as the signal-to-noise is limited by the quantal nature of the signal.) Paradoxically, it is the ease with which such weak optical signals can be recorded that may make them appear so noisy in comparison with electrical records.

2. General principles of fluorescence

Comparison with photoproteins and absorbance measurements

Fluorescence techniques are now very popular, and it is worth making a brief comparison with other older optical methods of intracellular ion concentration measurement. The first optical methods involved the measurement of dye absorbance (e.g. metallochromic indicators such as murexide, arsenazo or antipyrylazo) but this technique has several disadvantages. The main one is that cells are quite small so that the optical path along which absorbance is being meaured is quite short (< 1mm). This results in only a low level of absorbance for reasonable indicator concentrations, and the absorbance signal has to be separated from the signal due to light scattering as well as from intrinsic absorbance. In practice, the situation is greatly improved by measuring the absorbance changes differentially between a pair of wavelengths, which maximises the indicator-related changes and minimise the others. This allows small concentration changes to be measured with surprisingly high accuracy and time resolution (Thomas, 1982), but estimates of absolute concentration levels are much more uncertain unless in situ calibrations can be performed. On the other hand, since the source of the light is external rather than internal, the indicator is not consumed and so measurements can be carried out for quite long periods. Nevertheless, absorbance techniques have been mainly limited to large invertebrate cells or muscle fibres, since the average mammalian cell is at least 10 times smaller in linear dimensions, resulting in a considerable (approaching 100-fold) reduction in the absolute amplitudes of the recorded optical signals as well as in a 10-fold reduction in the absorbance, which may preclude useful measurements. Further description of absorbance measurements is given elsewhere (see Ashley & Campbell 1979; Thomas, 1991).

Photoproteins (such as aequorin) are enzymes (mol.wt. 22,000) that catalyse the oxidation of a bound prosthetic group (such as coelenterazine-for review see Blinks et al., 1976). The oxidation reaction gives off a photon (as do many oxidation reactions) and the catalysis is regulated by calcium. The principal disadvantages of this technique for measuring calcium arise from the difficulties associated with

introducing the photoprotein into the cell. Furthermore, on average, less than one photon is emitted by each photoprotein molecule (typically, only one molecule in three releases a detectable photon). In addition, the rate of photon emission has a highly nonlinear calcium dependence, which means that photoproteins tend to overestimate the spatially averaged calcium concentrations. Despite these disadvantages, the technique has a major advantage over absorbance measurements in that the detected signal arises only from the injected photoprotein, so movement artifacts and light scattering are not a significant problem. The light emission at a given calcium concentration depends on the the amount of active photoprotein in the cell, but it is possible to take this into account by measuring the amount remaining in the preparation at the end of the experiment by using a non-ionic detergent to make the cell leaky to calcium. The signals obtained during the experiment can then be expressed as fractions of the amount of active indicator, and converted to calcium concentrations by relating them to in vitro calibration curves (Allen & Blinks, 1979). In some circumstances, a significant proportion of the photoporotein may be consumed during the experiment, but it is possible to correct for this, by expressing the luminescence at any given time as a fraction of the total luminescence remaing at that time. It was the success of the photoprotein technique that revolutionised the field of calcium metabolism in cardiac muscle cells (volume approx. 30 pl), but the difficulties associated with handling and injecting these sensitive calcium indicators have discouraged their widespread use. More recently, the prospect of expressing genetically engineered photoprotreins in situ (Rizzuto et al., 1983) could mean that the technique is on the verge of a renaissance (although the enzyme system that builds the coelenterazine has not yet been cloned and cells have to be incubated with it overnight).

Fluorescent indicators combine advantages from both of the above methods. The fluorescent indicators can be considered to be self luminous (and therefore provide the advantages of the photoproteins) without concern for loss of activity on exposure to calcium. Conventional organic synthesis allows the properties of the indicators to be modified (by, for example, adding dextran residues to increase molecular weight and reduce indicator compartmentalisation and excretion from the cell), and since each fluorescent molecule can emit many photons the signal to noise ratio is high. It is notable that calcium signals have been recorded from cells as small as platelets with the newer calcium indicators. The fluorescent molecule (or fluorochrome) is usually excited by the absorption of a photon. This raises the energy of the molecule to a new "singlet" state from which the molecule descends the vibrational ladder until a radiative transition takes place and the molecule returns to the ground state with the emission of a photon. Alternatively, the photon emitted may be reabsorbed or the excited state may be quenched by collision with another molecule. In any case, the number of emitted photons are somewhat less than the number of absorbed photons and the ratio between them is called the quantum efficiency (modern fluorochromes have quantum efficiencies of about 0.3). The energy of the emitted photon is ordinarily lower than that of the absorbed photon, so its wavelength is correspondingly longer. However, under conditions of very high light intensity, i.e.

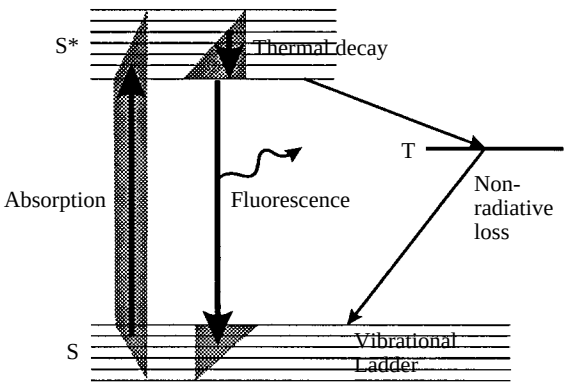

Fig. 1. An energy level diagram for fluorescence excitation. Note the presence of multiple vibrational levels around the principal energy levels S and S*.

pulsed laser illumination, it is possible for the indicator to absorb two long-wavelength photons in rapid succession, simulating the absorption of a single photon of half their wavelength. In the high energy state, the molecule is more likely to be oxidised and this will result in a loss of fluorescence (bleaching) but modern fluorochromes are also quite resistant to this effect. If the excitation and emission wavelengths are sufficiently far apart, the exciting light can be blocked from the detector with suitable filters so that the emission can be measured against a dark background. In practice, the background will not be completely dark, as other cell constituents will also fluoresce to some extent. This is known as autofluorescence, but at visible wavelengths its magnitude is relatively small and it is often ignored (not necessarily with good justification).

Fluorescence excitation and emission spectra

A potentially confusing aspect of fluorescence measurements is that there are not one but two sets of spectra to consider. First, some wavelengths will be more effective than others in exciting the fluorescence, and this dependence can be quantified by measuring the fluorescence excitation spectrum (there are multiple excitation wavelengths because of the large number of vibrational energy levels associated with the fluorochrome molecule). Second, the fluorescent light will be emitted over a range of wavelengths (due to the vibrational ladder associated with the excited and ground states - see Fig. 1). Ion sensitive probes are made by attaching groups that bind ions to the fluorescent part of the molecule. The binding of an ion alters the electronic configuration of the molecule and hence alters the fluorescence of the molecule. For example fluo-3 has a calcium co-ordination site based on the BAPTA molecule and the fluorescent group (based on fluorescene) is attached to one side of the BAPTA backbone. Calcium binding to fluo-3 draws electrons from the BAPTA rings, which in turn draw electrons from the rings of the fluorescene group and thereby increase the fluorescence of the molecule (this explanation is something of a simplification as it is really the resonance of the ring structures that is altered).

In order to measure ion levels with such a fluorescent indicator, all one has to do is measure fluorescence at a suitable wavelength (both for excitation and emission). However, the raw signal would not be quantitative because the absolute fluorescence will depend on (1) the concentration of indicator, (2) the volume of the cell (or path length which is being illuminated), (3) the intensity of the illumination, (4) the properties of the detection system, (5) the cell autofluorescence, and finally (6) the calcium concentration (which is the only variable of real interest). However, variables 1-5 can (in principle) be eliminated by recording the maximum fluorescence of the cell at saturating ion levels and minimum fluorescence in the prescence of a quenching ion (such as manganese) or in the absence of the ion at the end of the experiment (see below).

Spectral shifts and ratio measurements

It is also possible that binding of the target substance by the fluorescent molecule will cause a shift in the fluorescence spectra rather than a simple modulation as discussed above for fluo-3. In principle, this concept is straightforward enough, but consideration of it is complicated by the further possibility that either the fluorescence excitation spectrum or the fluorescence emission spectrum - or indeed both - may be shifted. For example, fura-2 and indo-1 also have a calcium coordination site based on the BAPTA molecule with a fluorescent group attached to it. The alteration of electronic structure on calcium binding has different effects on these molecules in that for fura-2 the excitation spectrum shifts to shorter wavelengths, while for indo-1 the emission spectrum shifts to shorter wavelengths (see Fig. 2). Such shifts are desirable because they allow the concentration of the ion of interest (in this example calcium) to be estimated from the relative levels of fluorescence measured at two different wavelengths. This technique is known as ratiometric fluorescence measurement and is described in detail below.

The most important point concerning these spectral shifts is that they must be large enough to be detected easily, ideally in a part of the spectrum that does not require very specialised detection equipment (i.e. it is easier to design instruments to work in the visible wavelength range than in the far ultraviolet or infrared range of wavelengths). Although one can argue about which type of shift is the best on theoretical grounds, one also requires a reasonable affinity and selectivity for the target substance, high fluorescence quantum yield, lack of biological side-effects, and molecular stability. These other factors all tend to be just as important, so in practice it is best to design instrumentation that is flexible enough to allow use of a range of fluorescent indicators.

From the measurement point of view, the case where the emission spectrum shifts is the simpler one to deal with. The consequence of a shift in the emission spectrum is that the fluorescence at some wavelengths will increase, whereas at other wavelengths it will decrease, as shown in Fig. 1B. Although it is useful for calibration, and some other purposes, to measure the complete spectra, the parameter that is of greatest interest in biological measurements is the time-dependence of the fluorescence change. In practice, therefore, one normally measures signals at just two

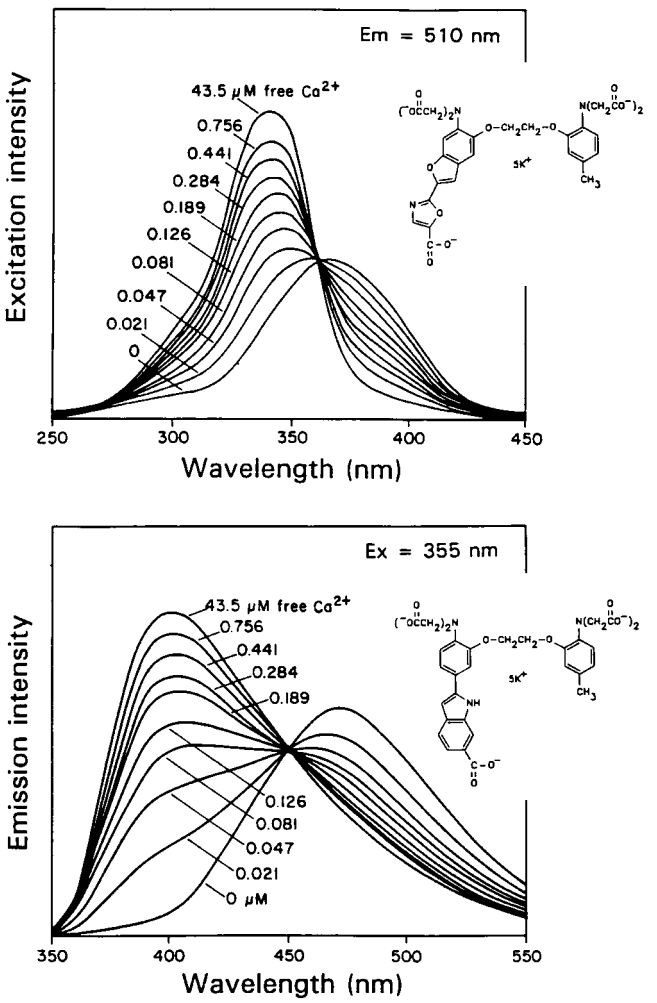

Fig 2. A shows excitation spectra for fura-2 with emission monitored at 510 nm. Note that with illumination at 340 nm the fluorescence increases with increasing calcium, while at 380 nm the fluorescence decreases with increasing calcium. B shows emission spectra for indo-1 with excitation at 355 nm. Note that as calcium increases the emission spectrum shifts to shorter wavelengths. The traces labelled F were obtained at 0.1 μM free calcium, a level comparable to resting levels in the cell. Data redrawn from Grynkiewicz et al., 1985.

wavelengths - or to be more precise, two waveBANDS - so that as many of the emitted photons as possible can contribute to the measurements to maximise the signal-to-noise ratio. Thus, all one needs to do is split the output signal and pass it to two photodetectors, preceded by optical filters of suitable characteristics to define their spectral sensitivity. The optical efficiency of the arrangement can be improved by using a dichroic mirror to split the output signal as this will maximise the signal to each detector (dichroic mirrors reflect light of some wavelengths and transmit the rest, so by placing one in the light path at an angle of 45 degrees, the emission light

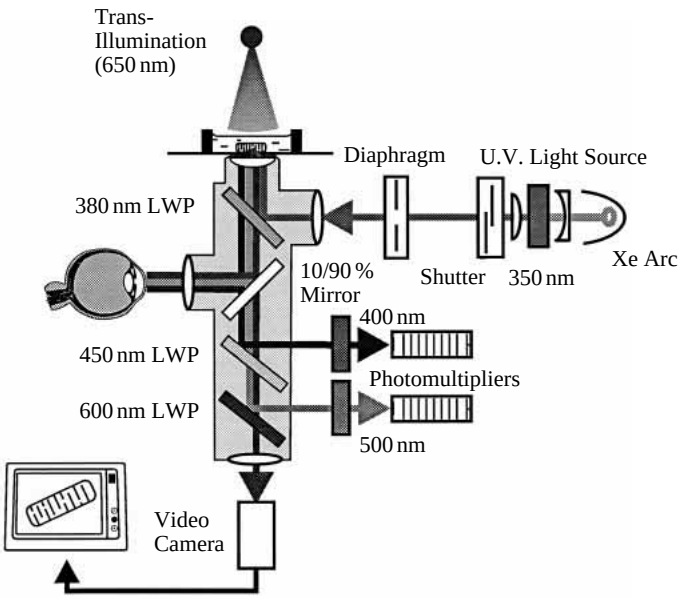

Fig. 3. A schematic diagram of an inverted epifluorescence microscope system for indo-1 fluorescence detection. For fura-2 this system would have to be modified to include some method for changing excitation wavelength. The LWP (long wave pass) mirrors are dichroic mirrors that split the light at the wavelengths indicated. Note that red light (650 nm) is used to give continuous illumination of the preparation, so that it can be observed by eye or via a camera.

can be steered to one detector in the straight-ahead position or to another one at 90 degrees according to its wavelength; see Fig. 3).

The case where the excitation spectrum shifts is somewhat more complicated, because the emission spectrum will (at least sometimes!) remain the same. To measure changes in the excitation spectrum, the individual excitation wavelengths must be supplied sequentially. This requires some form of optical chopping system, combined with some appropriate electronics on the detector side to separate out the fluorescence emission signals that have been detected for each of the excitation wavelengths. Clearly the time resolution of the system will depend on the chopping frequency, but other factors, such as the signal-to-noise ratio of the detected signals (which increases as the signal bandwidth increases), and the response times of the indicators, impose the ultimate limits, since it is relatively easy to perform the chopping on a millisecond timescale. Fortunately, the fluorescence lifetimes of all the indicators are very much shorter than this, so in practice there is no risk of cross-contamination between individual excitation wavelengths when operating at such speeds.

Multiple-excitation wavelength systems are more complicated, but in practice, much of the complexity is hidden from the user; indeed they may even appear simpler since only one detector is required. However, for a truly general-purpose instrument, the addition of a second photodetector allows dual-emission measurements to be

made as well. It is also possible to make measurements using two or more indicators at the same time, if their excitation and/or emission spectra are sufficiently different. For example, simultaneous measurement of Ca^{2+} with fura-2 and pH with BCECF has been carried out successfully, since fura2 is excited at 340 and 380 nm, and BCECF is excited at 440 and 490 nm. These excitation wavelength pairs each have little effect on the other indicator, whereas they both emit in the 500-550 nm range and so they can be monitored with the same photodetector (see Fonteriz et al., 1991). However, the experimental difficulties inherent in using two indicators simultanoeusly should not be underestimated.

3. Detection of optical signals

Two basic types of photodetector are in general use. These are photomultipliers and solid-state photodiodes, and they are based on vacuum-tube and semiconductor technology respectively. This is one of the few areas in which semiconductor technology has not displaced vacuum tubes.

Photomultipliers

A photomultiplier consists of a evacuated glass tube with a photocathode, an anode and a series of about 10 intermediate electrodes known as dynodes between them (see Fig. 4A). The photocathode is so named because it is coated with a material which emits an electron in response to the absorbance of a photon. Although the energy carried by a photon at optical wavelengths is relatively small (about 2 eV), it is sufficient to liberate an electron from the materials coating the photocathode. The electron is accelerated towards the first dynode, which is held at a more positive potential (on the order of 100 V), so it gains an equivalent amount of energy in eV on the way. When the accelerated electron hits the first dynode it liberates further electrons, which are then accelerated towards the next dynode in the series. This process repeats through the series of dynodes (each of which is about 100 V more positive than its predecessor) until the anode is reached, where an electrically detectable pulse appears when the avalanche of electrons arrives. Thus the single electron liberated by the photon has been multipled by several million by the secondary emission throughout the dynode chain. (It should be noted that no earthed metal should contact the surface of the tube or emitted electrons will be attracted to the glass of the tube and a large increase in dark current will result). The pulse at the anode only lasts a few nanoseconds because the electron transit time down the tube is relatively constant. The numbers given here are necessarily approximate because they depend on the type of photomultiplier and on the total supply voltage, but the important point is that photomultipliers can give such enormous amplification that the output signal is well above the noise level of the subsequent electronics. An important caveat to varying the supply voltage to the photomultiplier (and hence its gain) is that the cathode to first dynode voltage should ideally be kept constant. In particular, if this voltage is reduced too much, the energy gained by the first emitted electron may

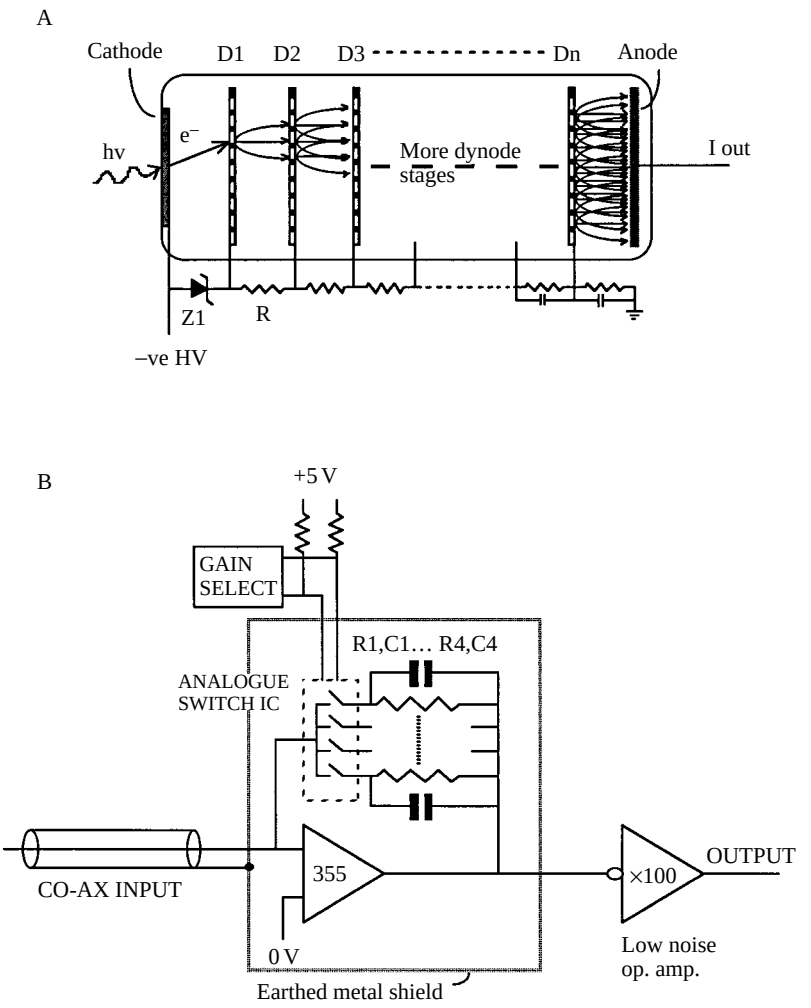

Fig. 4. A shows a schematic representation of a photomultiplier tube. Note that the voltage to the dynode voltages are set by a resistor chain, with the first dynode to cathode voltage stabilised with a zener diode. B is a schematic diagram of a current to voltage converter suitable for photomultiplier tube current measurements (see the Microlectrode Electronics chapter for further information). The gain is selected by an analog switch which could be controlled by a computer. The second ×100 inverting amplifier should be a low noise type with a bandwidth similar to that set by the combination of R1,C1..R4,C4 (the cut off frequency being $1/(2\pi RC)$. Alternatively the bandwidth of this amplifier could be computer controlled to filter the light signal as needed (see text). The gain of this circuit is: 100×R Volts/Amp, typical photomultiplier gains would be 0.1-1000 nA/V, corresponding to R= 1 MΩ–10 kΩ respectively.

prevent secondary emission, which would result in a serious reduction in detector efficiency. Thus if the experimenter wants to alter detector gain by altering the photomultiplier supply voltage, then the first dynode to cathode voltage should be set preferably by a zener diode rather than by a resistor in the divider chain (see Fig. 4A).

The requirement for a relatively high supply voltage (approx. 1 kV) for the photomultiplier is a slight inconvenience, but the usual practice is to run the photocathode at a negative potential so that the anode can be at ground potential. This makes the detection electronics much more straightforward. Detection can either be analogue (by measuring the average anode current) or digital (by counting the individual current pulses). The preferred configuration for analogue detection is to connect the anode to the input of a virtual ground amplifier, which acts as a current-to-voltage converter (see Fig. 4B). The other practical inconvenience is that photomultipliers tend to be physically large, but that may also be an advantage since there is no problem in directing all of the light onto the detecting area (10-25 mm diameter).

Photomultiplier technology has a number of variants, and the one most relevant to fluorescence is the image intensifier. This device uses a similar type of photoelectron multiplication, but spatial information is retained by preventing the electrons from wandering within the tube - either by using hollow fibres coated with a secondary emitter, with the accelerating voltage applied at the ends of the fibres (called a micro channel plate intensifier or second generation intensifier) or by using shaped electrodes within the tube to form an electrostatic lens (a first generation intensifier). The anode is replaced by a phosphorescent screen so that accelerated electrons hit the screen and produce light. Thus an intensified image appears at the end of the tube which may then be viewed directly (as in military image intensifier goggles) or else with a conventional TV camera. Just why such a device may be useful will become clear in the following discussion on photodiode technology.

It should be noted that photomultipliers must always be protected from bright light when powered, as permanent damage to the tube can occur if the anode current rises above the limit set by the manufacturer (although the high-voltage power supply may incorporate current-limiting circuitry to reduce this risk). Following exposure to bright light (even with the power off) the dark current of the tube may be increased (James, 1967), an effect that may decay over several hours if the tube has not been damaged. However, damage to the photocathode may permanently reduce the sensitivity of the tube, an effect that can only be determined if the experimenter has a light standard with which to test the tube. (A simple light standard can be made from a light emitting diode run from a stable current source. The intensity of the light source can be reduced by painting the surface of the diode with black paint and the diode should be mounted in a holder that fixes the geometry of the source.)

Photodiodes and CCD cameras

A photodiode is a single-point detector and is the solid state analogue of the vacuum photodiode. A CCD camera basically consists of an array of photodiodes, and although there are differences in detail, the underlying principles are similar, so the case of a single photodiode will be considered here. In these devices, the absorption of a photon causes charge separation (more specifically, the formation of an electron-hole pair). This process is highly efficient, and the quantum efficiency approaches unity for wavelengths close to the bandgap energy of the semiconductor,

i.e. in the infrared at around 900 nm, but it is also high over most of the optical spectrum (in contrast, photomultipliers struggle to achieve much more than 25% quantum efficiency). If the diode is open-circuited, the charge separation will appear as a voltage across the capacitance of the device, or if it is shortcircuited by connection to a virtual ground amplifier, the charge separation can be measured directly as a current. This is the preferred method of operation for a single photodiode, since the response is linear over many orders of magnitude when measured in this way. However, unlike the photomultiplier currents, these photocurrents have not been amplified, so they must be measured against the thermal noise background of the device, plus that of the amplifier to which it is connected. In practice, the noise characteristics of the photodiode swamp those of the amplifier, since the primary noise source is the leakage resistance that appears in parallel with the device, which typically has a value of around 100 megohms for a photodiode of 1 mm^2 active area (see the application notes for the OPA101 in the Burr-Brown Data book, 1982, for a detailed analysis of this configuration). In a CCD camera, the leakage resistance per pixel should be at least 10,000 times higher, as the pixel area is correspondingly smaller, i.e. the linear dimensions are about 10 μm instead of 1 mm, which then places greater demands on the noise performance of the amplifier because the signal per pixel is correspondingly smaller. Since the current is measured by a current to voltage converter the noise limits are the same as those of the patch clamp amplifier (described elsewhere), with the same dependencies on source resistance and amplifier noise. For detailed treatment of noise see the next section, but *assuming* that it is possible to detect about 1 pA photocurrent at 1 kHz in a small photodiode (<1 mm^2), this represents a lower limit for detection of about 6,000,000 photons/s. Since this implies 6000 photons per measurement bin (a photomultiplier might give no more than one spurious count per bin at this bandwidth) the superiority of the photomultiplier is obvious (although the reduced quantum efficiency would result in an ouput of about 10^6 counts/s the photomultiplier output would be almost all signal (signal to noise ratio = 1000) whereas the diode output would be almost all noise). One way that the "sensitivity" of silicon diodes can be improved is by allowing the charge to accumulate over a period of time until it is large enough to be reliably read out. However, to allow long integration times the detector must be cooled to prevent thermal energy from destroying the electron-hole pairs as well as contributing to Johnson noise (see below) (astronomers use liquid nitrogen cooling and sometimes integration times of more than an hour!). In arrays of photodiodes (which make an imaging sensor), it is impractical to connect each pixel diode to its own amplifier, and instead the charge separations are accumulated within each pixel diode and then sent in turn to a single amplifier via a shift register arrangement (hence the term Charge Coupled Device for this type of photodetector). Unfortunately, the charge readout and reset process introduces additional noise. Nevertheless, total detector noise levels of only a few photoelectrons per pixel can be achieved from the best CCD cameras currently available, and there may still be some scope for further improvement.

To extend the light range over which CCD cameras operate a microchannel

intensifier (see above) can be optically coupled to the sensor (by lenses or with a coherent fibre optic plug). Such intensified CCD (or ICCD) cameras have become very popular for fluorescence imaging. However the dynamic range of these cameras (at a given gain) is not as large as photomultipliers, and saturation effects are easily observed. The experimenter should therefore ensure that the camera gain is adjusted so that the majority of the image field is captured within the linear part of the camera response - a precaution that applies to both images when ratio imaging. Put another way, the average ratio in a field should be near unity, a condition that can be obtained by altering the intensity of excitation wavelengths appropriately.

In summary therefore, although photomultipliers have lower quantum efficiencies, they allow the detection of individual photons (albeit one in 3 or 4), which is not possible with silicon technology. Silicon devices are only useful if the total number of photons received within the measurement period exceeds a limit set by the read out electronics, so that the photomultiplier with its large detection area remains a superior choice for the majority of low light level fluorescence measurements.

Noise in detectors

In this section we must distinguish between THERMAL (Johnson) noise which is generated by the random movement of electron in conductors and SHOT noise which is due to the statistical variation in the rate of arrival of particles (photons or electrons). This is important because in photodiodes, both shot noise and thermal noise will contribute to the signal whereas in photomultipliers the enormous current amplification that occurs in the dynode chain ensures that only the shot noise is important.

Since noise generation is a random process, the individual noise sources add their contributions in quadrature, i.e. the total noise is the square root of the sum of the squares of the individual components. This also applies to the bandwidth over which the noise is measured, so if the noise is equal at all frequencies, it increases with the square root of the bandwidth. Therefore noise is typically specified in units of volts (or amps) per Hz. The thermal noise also varies with the square root of the resistance. The equation for the noise voltage (V_n) across a resistance R is:

$$V_n = \sqrt{4KTR\Delta F}$$

(where K is Boltzmann's constant (1.38×10^{-23} J per degree), T is absolute temperature and F is the bandwidth in Hz). Assuming a temperature of 25°C we can write:

$$V_n/\sqrt{Hz} = 1.28\times10^{-10}\sqrt{R}$$

Thermal noise is usually expressed as a voltage but we are more interested in current noise (I_n) which from Ohm's law is given by:

$$I_n/\sqrt{Hz} = 1.28\times10^{-10}/\sqrt{R}$$

This is to be compared to the shot noise, which is given by:

$$I_n = \sqrt{2eI\Delta F}$$

where e is the charge on an electron (1.6×10^{-19} coulombs) so

$$I_n/\sqrt{Hz} = 5.66\times10^{-10}\sqrt{I}$$

The two components will be equal when:

$$1.28\times10^{-10}/\sqrt{R} = 5.66\times10^{-10}\sqrt{I}$$

i.e. when

$$I=1/19.64R$$

Note that this relationship is independent of the measurement bandwidth, since both noise components increase with the square root of the bandwidth. Thus for the shot noise current (which increases with the square root of the current, i.e. light intensity) to equal the thermal noise in a 100 megohm resistance of our typical photodiode, the current itself must be:

$$I = 5.09\times10^{-10} \text{ A or } 3.18\times10^{9} \text{ (photo)electrons per second.}$$

It is therefore clear that such a photodiode is no match for the photomultiplier when it comes to detecting low photon fluxes.

Actually this discussion is relevant to any current measurement where all the thermal noise (including amplifier noise) can be modelled as a single resistance. This

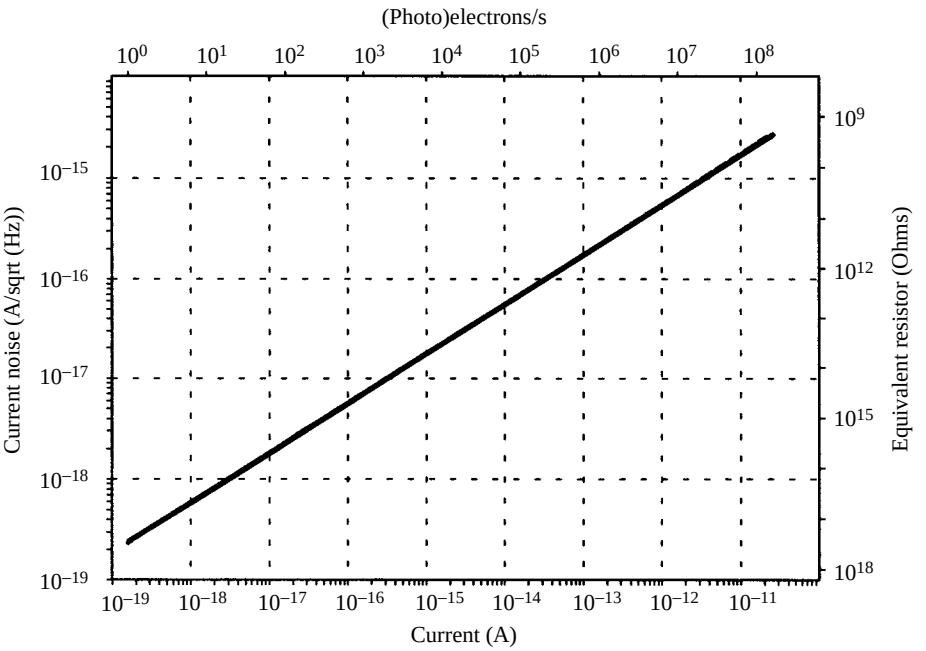

Fig 5. The relationship between current flow and shot noise at 25°C. The top of the graph shows the electron flow rate and the value of the resistor that gives the same noise level is shown at the right axis. Note the large resistor values that would be required to reproduce shot noise.

is illustrated in Fig. 5 where the current is shown on the x-axis both as a current (bottom) and as an electron flow (top) and the associated noise is shown on the y-axis. The resistor that would give the equivalent noise is shown on the right. Thus if the background noise from a photomultiplier is modelled as an equivalent resistance (this is perfectly legal) we see that at a typical value of 100 counts per second, the noise is equivalent to that from a resistor of about 3×10^{15} Ω. For a good photomultiplier, cooled to 0°C, a noise level of 1 count per second can be achieved, giving an equivalent resistance of about 3×10^{17} Ω. These figures make the gigaohm resistances used in patch clamping look quite pedestrian, and serve to emphasise just how sensitive optical measurements with photomultipliers really are!

In a CCD image sensor, the pixel area (photodiode) may be much smaller and a resistance of 10^{12} Ω per pixel might be realised. However, this does not reflect a real improvement in "sensitivity" because the light flux is spread over the whole sensor area and so the number of photons arriving at each pixel falls (in direct proportion to the pixel area). However, if the experimenter reduces the magnification of the image (keeping other factors constant) the sensitivity will improve (because the photon flux incident on each pixel will increase). Thus the image magnification should be kept as low as possible, consistent with the desired spatial resolution, to maximise signal to noise in a fluorescence image.

As noted earlier, cooling helps improve CCD performance. This arises because (1) the Johnson noise is proportional to the square root of the absolute temperature (this is not a large factor unless liquid nitrogen temperatures are approached) and (2) the resistance of the pixel elements increases as the temperature is reduced. This increase in resistance also allows the image to be acquired over a longer period, since it reduces the rate at which charges leak away. Taking advantage of this effect to reduce the measurement bandwidth will reduce the noise still further. The other important noise sources are the noise in the detecting amplifier and the charge shift/read out electronics, in which the noise will be reduced by reducing the bandwidth. These effects make cooled CCD cameras very suitable for long term observation of dim objects but are rather less well suited to the relatively rapid changes in fluorescence encountered in biophysics. Despite the advantages of cooling CCD sensors, they will not be able to detect single photons.

Despite these criticisms, it is important to note that for shot noise levels, above thermal levels, photodiode detectors will be superior to photomultipliers, because their quantum efficiency is much higher. For example, in absorbance measurements photodiodes are the detectors of choice, and one of the authors (M.V.T.) found that for absorbance measurements on molluscan neurones, the shot noise was indeed the dominant noise source in a detector circuit using a 1mm photodiode.

In conclusion, shot noise will always limit the signal to noise ratio for a perfect detector, thus there can be no substitute for efficient collecting optics. In practice it may be easier to improve collection efficiency rather than squeeze the last drop of performance from the detector system. Nevertheless, the correct choice of detector and its proper implementation can be crucial for experimental success.

Analogue detection or photon counting?

There tends to be a certain amount of confusion concerning photomultiplier signal detection methods, particularly with regard to the possible advantages of the photon counting technique over analogue detection. This section gives a detailed comparison between the two methods, so that the advantages and drawbacks of each can be fully appreciated. However, for readers who just want to know which method is the better one, the answer (in the authors' opinion) is that for typical applications analogue circuitry is perfectly adequate (and is simpler than photon counting). For analogue signal detection, one just measures the average anode current, whereas photon counting uses a discriminator circuit, which provides an output pulse when the input current rises above a threshold value. Since the current pulses are not instantaneous, they can only be recognised reliably as single pulses when the interval between them is large compared with their individual duration. As the light level increases, the pulses become closer together until they can no longer be individually recognised, and the detection efficiency will fall. Analogue circuitry does not exhibit this problem as the anode current rises in proportion to the light intensity until tube saturation effects occur. Furthermore, the light level at which tube saturation starts can be increased by reducing the supply voltage (but see caveat above). Photon counting is thus possible only for relatively low light levels, whereas analogue detection is possible at any light level.

For very low light levels, photon counting does has definite advantages, since (1) the output pulses are of constant amplitude and duration (2) the output signal in the absence of light is lower because of the rejection of (i) thermally generated, (ii) cosmic ray and radioactive decay generated events. At a given tube operating voltage, the amplitude of a current pulse caused by a photon arriving at the photocathode occurs within a relatively narrow range, so the discriminator threshold can be set just below it (in practice, it may be more convenient to set the current pulse amplitude to match the discriminator threshold, by varying the photomultiplier supply voltage - but see above). When this is done, the discriminator rejects most of the thermally generated pulses (thermionic emission occurs from the photocathode and dynodes; however only thermally emitted electrons arising at the photocathode will cause a full size output pulse whereas those occurring within the dynode chain cause smaller pulses because the number of amplification stages is reduced). In addition, extra large pulses resulting from cosmic rays or radioactive decay particles hitting the tube may be rejected by the discriminator (or else are simply reported as normal photon pulses). While the rejection of these spurious pulses might seem a real advantage, in practice the number of large pulses is small (1 per second) and the quite large number of thermionic pulses does not cause as large a dark current as might be expected since they are of reduced amplitude (see Sharpe, 1964). As a practical example, one of the authors (M.B.C.) uses EMI 9789 tubes, which have a dark current of about 0.1 nA. The same tubes used in a typical fluorescence experiment give a signal of about 50 nA showing that the tube dark current is negligible.

For biological luminescence measurements, where only a few photons may be detected per second, the use of photon counting may be considered essential, but it

should be noted that greater improvements may be made by building a cooled housing for the photomultiplier tube (cf. Cannell & Allen, 1983) to reduce the dark current. Only when this has been done is it worth contemplating the extra complexities of photon counting.

Photon counting can offer an improvement for signals in the biological fluorescence range, but the effect is quite small. It arises because there is a statistical variation in the amplitude of the current pulses arriving at the anode, which results mainly from the variation in the number of electrons that are emitted by the first dynode as a result of the impact of the photoelectron (the same effect occurs at all the other dynodes too, but what happens at the first dynode is the predominant source of the variation). For analogue detection, this variation causes an additional noise component, but for well-designed tubes the effect should increase the total noise by no more than about 10-20%. The relative size of the variation can be reduced by applying a higher accelerating voltage to the first dynode, causing each photoelectron to emit a larger number of electrons from it, and the voltage divider networks supplied with commercially available tubes usually incorporate such an arrangement.

This improvement may be worth having, although one has to consider the loss of information that can occur with photon counting on account of the dead time. The main problem with photon counting is that two coincident or closely spaced pulses cannot be resolved as separate events, whereas they would both be recorded by analogue detection. The minimum interpulse interval for both pulses to be recognised by photon counting is known as the dead time, and it is normally set to a known figure by design, so that a statistical correction can be applied to the data to compensate for the undetected pulses. The correction is simple. If we detect N pulses in time T, and the dead time is given by t, then the count will actually have been obtained in a time T–Nt instead of T, so to compensate for the lost pulses we can estimate the true count N′ to be $N' = N \times T/(T-Nt)$. However, we cannot compensate for the loss of signal-to-noise arising from the loss of those pulses. Typically t is in the range of 100-500 nanoseconds, so to take a typical example with t = 200 nsec and N = 100,000 per second, then the true count N′ will be underestimated by about 2%. This is clearly not very significant, but for count frequencies approaching 1 MHz the losses become more serious, to the extent that analogue detection would be the better choice on theoretical as well as practical grounds.

To offer a similar word of caution on the analogue side, it is quite easy to devise an analogue detection system that performs nowhere near as well as it should, and claims that photon counting is far superior may well have been based on comparisons with such systems. The best method of analogue detection is the direct analogue equivalent of photon counting, i.e. to use an integrator to store the total charge that arrives within a given interval. At the end of the measurement period, the integrator output is digitised for data processing by computer, and/or stored in a sample-and-hold amplifier to give an analogue output that remains at a steady level until the next measurement is made. The integrator output is then set to zero in preparation for making the next measurement. A simpler technique is to use a current-to-voltage converter, with its bandwidth limited by a capacitor across the feedback resistor. This

can also be viewed as a "leaky integrator", in which the continuous discharge pathway presented by the resistor removes the need for discontinuously discharging the capacitor (see Fig. 4). However, as with the any other analogue signal, if it is to be digitised, then a sampling rate appropriate to the signal bandwidth must be chosen. Too low a sampling rate will result both in loss of information (i.e. reduced signal-to-noise ratio) and in the generation of additional noise components via the phenomenon of aliasing. The integration method method allows the integation time (and hence the effective bandwidth) to be altered in step with the sampling rate so that the bandwidth is always appropriate for the sampling frequency. An additional advantage is that in multiple-excitation systems, the integration period can be made to correspond exactly to the period during which a particular light filter is in the excitation path. This eliminates the possibility of contamination by previous signals from other filter wavelengths.

To summarise, photon counting is only really needed if the experimenter anticipates detecting very low light signals (i.e. those that are not visible to the dark adapted eye). It may actually degrade the signal if the discriminator is improperly adjusted or if large signals are to be measured. Analogue circuitry is simpler and is more likely to perform at or near its theoretical limits. It is certainly the preferred method for those who wish to construct their own equipment.

4. Instrumentation

Light source

Many of the currently available fluorescent indicators need to be excited in the near-ultraviolet, e.g. around 360 nm for the Calcium indicator indo-1, and 340-380 nm for the calcium indicator fura-2 and the sodium indicator SBFI. These requirements make fluorescence instrumentation somewhat more expensive, as incandescent light sources (e.g. quartz-halogen) do not give sufficient light at these wavelengths, so an arc source is all but essential. The almost universal choice is xenon, as this gives a very uniform output spectrum, which extends between ultraviolet and infrared wavelengths. Mercury arc lamps are less suitable for this application, because their spectra are very irregular, giving huge outputs at some wavelengths and very little at others (see Fig. 6). However, this distinction is not usually relevant to the choice of light source, as most arc sources will take either type of bulb.

Arc sources produce high-intensity light with reasonable efficiency, but stability of the arc source with time is not as good as that of incandescent lamps. In addition, there is a problem with these sources that arises from the arc "wandering" between the arc electrodes. Since the arc colour and intensity are not constant over the area that the arc encompasses, quite large variations in illumination intensity (up to 40%) may result from using optical systems that depend on imaging the arc on the specimen (Levi, 1968). While this problem can be overcome by obtaining a feedback signal from the illumination to control arc current, some care should be taken to ensure that the arc has not wandered so far that the arc lamp is excessively

overdriven. In comparison, incandescent light sources are far more stable and are easily stabilised to less than 1% intensity variation.

Selection of illumination wavelength may be performed with interference filters or with grating monochromators (note that coloured glass filters do not have a rapid enough roll-off of transmission efficiency to be generally useful). While interference filters are relatively inexpensive and readily available, they have problems in their application that are not present in monochromators. The first is that attention must be paid to the incident energy load that is presented to the filter. Interference filters should not be used to select illumination wavelength in such a way that an appreciable heat load is placed on the filter; typical interferences filters will be permanently damaged in situations where their temperature rises to more than about 70°C. In practice this means that an interference filter should not be placed directly in front of an arc lamp source. Instead, unwanted light should be first absorbed with coloured glass filters or, better still, reflected from the optical path with dichroic mirrors (often called "hot" or "cold" mirrors and commercially available for this purpose). Interference filters are usually constructed so that their front surface reflects as much as possible of the unwanted radiation, which greatly reduces the amount of energy that they absorb, and so it is particularly important that they are installed in the correct orientation. Water filters are good at absorbing unwanted infrared light, but the white light that passes through them may still be intense enough to cause damage

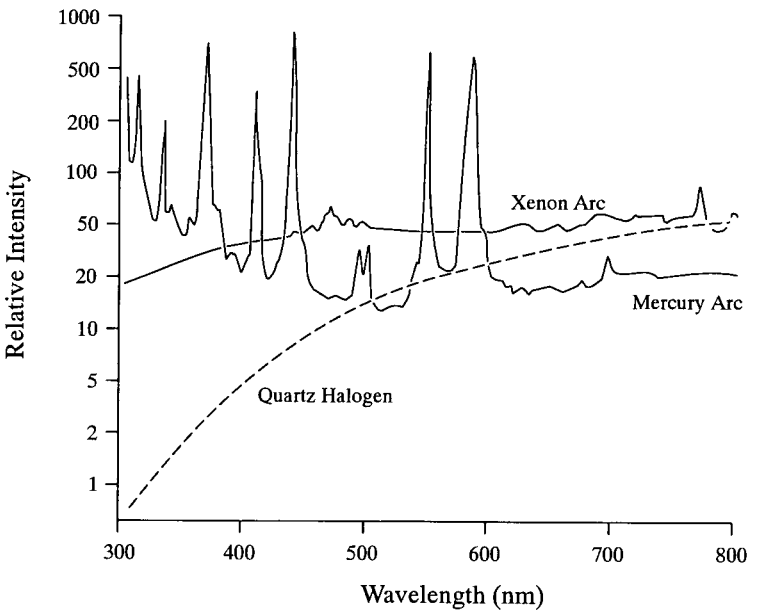

Fig. 6. Comparison of typical spectra of xenon, mercury and quartz halogen light sources. Note that the halogen source is the most uniform but the output drops quite rapidly in the violet and is not useful for UV illumination. The xenon arc source has a smoother spectrum than the mercury arc, but the sharp peaks of the mercury spectrum can be useful if they occur at desired wavelengths.

to the interference filter with time. A second problem arises from the pass-band of an interference filter being dependent on the angle of incident illumination, although for light within 0-10° of the optical axis the effect is relatively small. In practice this means that the light passing through the filter should be reasonably well collimated to achieve the manufacturer's specifications for the filter. Since the pass-band of the filter shifts to shorter wavelengths with increasing angle of illumination, the filter may be "tuned" to the desired wavelength by placing it at an angle to the optical path, but collimation becomes more important under these conditions, since the variation in the pass-band wavelength with angle of incidence increases considerably as the (average) angle is increased. Typical filters will shift to about 98% of the nominal pass band wavelength at an incident angle of 15°. Where the light is not collimated, this will result in a broadening of the pass band of the filter and an effective shift of the pass band to shorter wavelengths.

A growing number of indicators require excitation at around 490 nm (e.g. fluo-3 for calcium), for which an incandescent source would be perfectly satisfactory. Most of these indicators were introduced for confocal microscopy applications, where a laser must be used to obtain sufficient excitation intensity, and the argon laser at 494 nm is a good choice. Unfortunately, these indicators are generally not ratiometric, so they are not quite so attractive for other applications.

The other problem with the near-UV requirement is that ordinary glass has a very sharp transmission cut-off at around 350 nm, so all lenses in the optical excitation pathway must be of quartz or silica in order to allow fluorescence excitation at 340 nm. In practice, this does not seem to be a problem now that microscope manufacturers recognise the need for such lenses, but it does still rule out some very nice microscope objectives (e.g. some plan apochromats). Objectives that would otherwise be marginal or unsuitable can sometimes give satisfactory results if the excitation wavelength can be increased slightly, e.g. use of 350 nm rather than 340 nm for short-wavelength excitation of fura-2 will give more signal at the expense of a smaller range of ratios.

The optical system

The most commonly used optical system is the inverted microscope. This is particularly convenient for cell cultures or single-cell preparations, as individual cells can be illuminated and viewed from below, while preserving accessibility from above for simultaneous use of other techniques such as voltage recording or patch-clamping (see Fig. 3). In both this and the conventional (upright) microscope configuration, the fluorescence excitation light is supplied through a side port. A dichroic mirror situated behind the objective reflects the excitation light through the objective, which focuses the light onto the cell, thereby acting as the condenser lens. The objective also captures the emitted light, and the dichroic mirror is selected so that it transmits rather than reflects this light, so that it passes through the rest of the microscope. The numerical aperture (NA) describes the ability of the objective to capture light and is equal to $\mathbf{n.sin}\Theta$ where Θ is the half angle of the cone of light collected by the objective and **n** the refractive index of the medium between the specimen and the

lens. Most biological specimens are immersed in saline for which **n**=1.3. This represents the upper limit for the effective numerical aperture of the objective, so that even though higher numerical aperture objectives are made, they will be limited by the refractive index of the bathing medium. The amount of light collected (assuming the entire object is visible within the field) is given by the solid angle fraction $(1-\cos\Theta')/2$, where Θ' is the angle of the cone of light from the specimen. Θ' and Θ are related by Snell's law: $\sin\Theta'/\sin\Theta = n'/n$ where n and n' are the refractive indexes of the medium containing the specimen and the medium between the objective lens and the specimen medium. These equations suggest that a 1.4 NA objective will collect about 25% of the emitted light whereas a 0.8 NA objective will collect about 7%.

Clearly one should use as high a numerical aperture lens as possible for efficient detection and illumination. In fact the objective lens may be the most critical component for imaging and the experimenter would be advised to compare different lenses even if they have similar NA as the presence of field stops within the lens may reduce the light collection further. Some form of optical switching system is incorporated to allow light to be sent to the eyepieces for visual observation or to one or other output ports for photometry and/or camera viewing. For photometric measurements, it is also usual to provide an adjustable diaphragm, together with some method of viewing it, to select the area of view that is being sent to the photomultiplier(s). A particularly elegant way of doing this is to use deep red transmission illumination, to allow the selected area to be viewed with an inexpensive CCD camera. Since CCD cameras are very sensitive in the deep red/near-infrared (640-900 nm), the rejection of these wavelengths by the filters used to shape the fluorescence emission waveband is quite straightforward. Thus it is possible to view the preparation while recording very low levels of fluorescence.

A practical tip that can avoid a great deal of uncertainty over whether the equipment is working properly is that if a fluorescence signal is large enough to give a satisfactory recording, it should also be bright enough to be observed with the dark-adapted eye. This can be particularly informative when adjusting the conditions to give the best results when the ester loading technique is used to introduce the indicator into the cells.

For relatively large cells, for measurements in situ, or for experimements in cuvettes, a pair of fibre optic probes can be used to carry the excitation and emission light, or alternatively a single probe can be used in conjunction with a dichroic mirror to separate the two signals.

Detector arrangements

Continuously spinning filter wheels are a convenient (and commercially available) way of changing wavelength. These systems are usually available with all the necessary electronics to extract the relevant signals. They often allow up to six or eight filters to be used and the extra filter positions can be used to make measurements at additional wavelengths, and/or (as often happens) where the fluorescence from one wavelength is relatively weak, two or more filters can be

provided for that wavelength and the individual outputs can be combined electronically.

5. Using fluorescent indicators

Fluorescence is a rapidly developing field, and many further developments are likely to occur during the lifetime of this edition of the book. This section will therefore concentrate on the principles underlying the use of the fluorescent indicators, and the reader should consult current publications for the most up-to-date information on individual indicators. A useful source of additional information is the catalogue and regular updates published by Molecular Probes (see the references for their address).

Introducing the indicators into cells

The most reliable method of indicator loading is by direct injection through a microelectrode (Cannell et al., 1987, 1988). The injection technique is discussed elsewhere in this volume, so little needs to be said about it here, but of course it is limited to those cells that are large enough for application of such methods. However, indicator can also be loaded through the electrode when the cell-attached patch recording technique is used, which allows relatively small cells to studied.

Once introduced into cells, the fluorescent indicators generally remain there for a reasonable period, allowing stable recordings to be made. Even if there is some loss of indicator over time, the effects may be compensated for by the ratiometric measurement method. However, there are cases in which loss of indicator is a problem, and this effect can be reduced by injecting the indicator in a dextran-linked form. Linkage of the indicators to the dextran polymer does not in general seem to affect their properties significantly but inhibits cellular transport of the indicator (see the Molecular Probes catalogue for further information and for availability of these forms).

A particularly attractive feature of many of the fluorescent indicators is the possibility of introducing them into cells by the ester loading technique. This technique has extended fluorescence measurements into the realm of very small cells such as blood platelets. The fluorescent indicators are highly impermeant on account of being multiply charged at neutral pH, but on most indicators these charges are all carried on carboxyl groups. By esterifying these groups, an uncharged derivative of the indicator can be produced (Tsien, 1981). This derivative is not an active indicator, but it is sufficiently lipophilic to permeate biological membranes and thereby enter cells. Inside cells, the derivative is converted to the active indicator by the action of intrinsic esterase enzymes. Since the active indicator is impermeant, the effect is to cause accumulation of this form in the cells, so only a low concentration of the ester need be present in the external medium. This is useful, because the esters are highly insoluble (in fact they need to be dissolved in an appropriate carrier solvent before addition to the medium). Some care must be taken with this step, and the incubation conditions need to be adjusted carefully in order to achieve satisfactory loading of the

cells. There is also the risk that the indicator will be loaded into other cell compartments as well as into the cytosol (see below). A further potential problem is that the type of ester that needs to be used (acetoxymethyl) liberates formaldehyde as a hydrolysis product, although serious toxicity problems have not been reported.

The major problem with the ester loading technique is that the experimenter has little direct control over where the indicator ends up in the cell. The ester will enter all intracellular compartments and the active indicator concentration in each compartment will depend on the relative esterase activity. Thus the endoplasmic reticulum and mitochondria will also contain indicator that can confound interpretation of the signals. An additional problem is that de-esterification may be incomplete so that a fluorescent intermediate, which is not ion sensitive, may be produced (e.g. Highsmith et al., 1986). The magnitude of these effects can only be ascertained with careful control experiments, and at the very least the fluorescence from the cell should be examined under a microscope to ensure that it is relatively uniform.

A related problem is that some cells seem to be very good at clearing their cytoplasm of the indicator. In endothelial cells for example, after about an hour, the cell fluorescence appears punctate and mitochondria are clearly visible (M. B. Cannell, unpublished observations). Whether this represents simply the removal of cytoplasmic indicator or accumulation of indicator in the punctate regions is unclear at this time. In any case, considerable caution should be applied to the interpretation of signals from the cells loaded with the ester form of the indicator. (It should be noted that during the Plymouth Microelectrode Workshop we routinely observe that cells loaded with the ester give smaller ratio changes when depolarised to 0 mV than when the patch pipette loading method is used).

In summary, direct injection is always preferable to ester loading if there is a choice. However, good results can be obtained with the ester loading technique in many cases, provided adequate control experiments are performed. The ester loading technique may be the only route to take if cells cannot be loaded by patch pipette and it also has the advantage that many cells can be loaded at the same time allowing experiments to be performed on cell suspensions in cuvettes.

A few words on the handling of the indicators is in order here. The fluorescent indicators are all subject to oxidation during storage and will lose activity in a few days if exposed to light and air at room temperature. (It is for this reason that the indicators are supplied in ampoules sealed under argon or nitrogen). It is best to make up the acetoxymethyl esters in dry DMSO and split the indicator into a number of ampoules each of which contains enough indicator for a single experiment. The ampoules can then be frozen and this will avoid repeated freeze/thawing cycles. A similar procedure should be used for the free acid form of the indicator except that instead of DMSO a suitable buffer solution should be used (e.g. 140 mM KCl). The free acid form of the indicator should never be exposed to metal (such as stainless steel syringe needles). On the day of the experiment the aliquot of indicator is dissolved in about 1 ml of intracellular solution and placed in a 1 ml polyethylene tuberculin syringe, the end of which has been drawn into a fine capillary (to allow it to

pass down inside the electrode barrel). The syringe is used to place about 50-100 µl at the shoulder of the electrode, allowing a reasonable number of electrodes to be used before the syringe needs to be refilled. The experimenter should ensure that the small volume placed in the electrode is contacted by the silver wire of the electrode holder. Since the tip of the electrode is about 5 mm from the silver wire, diffusion of "silver contaminated" indicator should not be a problem. Finally, positive pressure must be maintained on the back of the electrode at all times before forming a giga-seal or else the experimenter will find that the indicator will be seriously diluted at the tip of the electrode (quite apart from the problem of contamination of the intracellular solution by bathing solution!). After breaking into the cell, dialysis of the cell may take 5-20 minutes, but the speed of dialysis can be markedly improved by applying brief pulses of positive pressure to the back of the electrode to "inject" the indicator (considerable care and experience is needed for this technique). The extent of dialysis can be determined from the magnitude of the resting fluorescence signal, and experienced patch clamp electrophysiologists may be amazed at how slow dialysis can be!

Interpretation of measurements

Although fluorescent indicators have proved to be extremely powerful, it is important to bear in mind that they share (as with all other recording techniques) the possibility of giving inaccurate measurements and/or of influencing whatever is being measured. In particular, it should be appreciated that the indicators work by reversibly binding to the target, so by definition they have a buffering action. The extent of the buffering will depend on the concentration of the indicator relative to the free concentration of the target ion as well as the affinity of the indicator, so it will be most significant for those ions whose free concentrations are relatively low, i.e. Ca and protons. Fortunately, the natural cell buffering capacity for these ions reduces the effect of indicator buffering. Nevertheless, the amplitudes and time courses of ion changes may well be significantly altered by the increased buffering, so one really needs to carry out experiments over a range of indicator concentrations to demonstrate that the results are reasonably independent of the indicator concentration. The binding equation is:

$$\text{fraction bound} = \frac{K_b[X]}{1+K_b[X]}$$

where

$$K_b = \frac{1}{K_d}$$

As an example, consider an experiment using fura-2 at 100 µM. Assuming a resting level of Ca and K_d of 150 nM, the fraction bound = 0.5. Hence, 50 µM Ca is bound to the dye and 0.15 µM is free. Thus the buffering power (B) = bound/free = 333.

Using another dye such as Furaptra: K_d = 44 µM and using 500 µM dye (very bright) gives B=10. These values can be compared to endogenous buffering powers

of 75 for immobile buffers and 20 for mobile buffers (Neher & Augustine, 1992), so that it is clear that the high affinity indicators can swamp the intrinsic buffers at usable concentrations. This will result in a reduction in the amplitude of calcium transients as well as a slowing in their time course.

Another problem is that the kinetics of the fluorescence change do not reflect the kinetics of the underlying calcium transient. This arises from (1) saturation effects in indicator response and (2) the kinetics of dye binding. While the first problem can be circumvented by converting the dye signal to ion concentrations (but see below), workers often assume that, because the dye:calcium stoichiometry is 1:1, the dye is linear and that the time course of fluorescence change should reflect the time course of calcium change. However this is not the case. From the equation:

$$\frac{dF}{dt} = \frac{d[X]}{dt} \cdot \frac{dF}{d[X]}$$

(dF/d[X] is the slope of the relationship between F and [X] -the ligand concentration) it is clear that we must consider the properties of the relationship between F and [X]. Initially, assume that the dye kinetics are so fast that the dye reaction is at equilibrium at all times (see below) and the binding is 1:1. Fig. 7 shows the relationship between F and dF/d[X] as a function of $[X]/K_d$ (the ligand concentration divided by the affinity of the indicator for the ligand). It is clear that dF/d[X]is considerably less than unity at [X] > 0.1Kd. This is the range within which most indicators are used, so that we

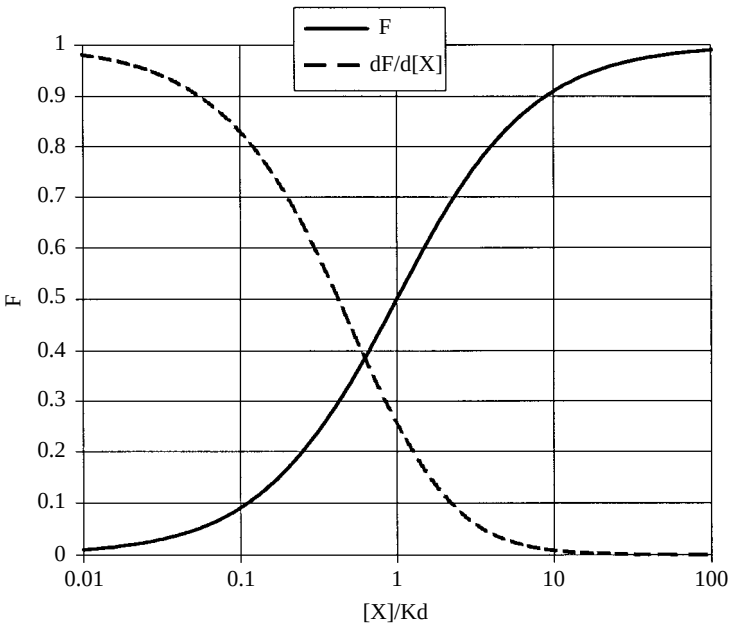

Fig. 7. The relationship between fluorescence (F) and ligand concentration ([X]) for a 1:1 stoichiometry. The ligand concentration is divided by its Kd; at the Kd 50% is bound. Note that the slope of the relationship between F and [X] is considerably less than unity around the Kd.

cannot take the time course of F as being the same as the time course of [X]. The simplest way around this is to convert F (or the ratio measurement) to [X] with a calibration curve.

However, dF/d[X] is also a function of time as the kinetics of the dye are limited. For the reaction:

$$D + X \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} DX$$

If the ion concentration undergoes a step change to [X] the indicator signal will change exponentially to a new level with a rate constant of ($k_{on}[X] + k_{off}$). For example, the kinetics of the fura-2 and indo-1 reactions with calcium have been investigated in stopped-flow experiments (Jackson et al., 1987). The rate of calcium binding by fura-2 is $2.5\text{-}6.5\times10^8$ M^{-1} s^{-1} and the rate of dissociation is 84 s^{-1} at 20°C. Indo-1 is slightly faster, with the rate constants of $0.5\text{-}1.0\times10^9$ M^{-1} s^{-1} and 130 s^{-1}, respectively. As might be expected, the rate of calcium binding is probably diffusion limited, while the lower affinity of indo-1 is reflected in the slightly higher rate of calcium dissociation. Thus at a level of 200 nM free calcium, the apparent fura-2 reaction will be about 180 s^{-1}. While this response speed is clearly superior to that of calcium-selective microelectrode and at least as fast as aequorin, it is not so fast that possible kinetic distortion of the calcium signal can be ignored. It is notable that Baylor and Hollingworth (1988) reported that the fura-2 kinetics appeared to be considerably slower in single frog muscle fibres than measurements of Jackson et al. (1987) would suggest. Baylor and Hollingworth (1988) compared the antipyrylazo absorbance signal to the fura-2 signal and suggested that the fura-2 signal might be explained if the fura-2 off rate were about 25 s^{-1}, a value three to four times lower than expected from *in vitro* experiments. The exact effect of the intracellular environment on the properties of fura-2 and indo-1 are still unknown. However, both Williams et al. (1985) and Baylor & Hollingworth (1988) reported that the fluorescence spectrum of fura-2 inside single smooth muscle cells was very similar to the spectrum obtained in a cuvette. In addition, although Williams et al. (1985) were loading the cells with the esterified form of fura-2 (which may have suffered from partial de-esterification problems - see above), they found that the calibration curve obtained by ionomycin exposure (to control intracellular calcium) agreed reasonably well with that obtained in a cuvette. It is notable that Williams et al. (1985) and Weir et al. (1987) found that the maximum ratio obtained from the intracellular dye appeared to be slightly less than that obtained in calibrating solutions, which suggests that the intracellular milieu alters the properties of the dye. A reduced coefficient for diffusion of fura-2 was observed by Baylor & Hollingworth (1988), an effect also found by Timmerman and Ashley (1986) in barnacle muscle. Fluorescence anisotropy also suggested some dye immobilisation (Baylor & Hollingworth, 1988). These results may all be due to the dye binding to some intracellular constituent, but whether such binding seriously alters the calcium sensitivity of the dye remains unclear.

Another potential difficulty concerns the fact that any indicator will give best resolution over a fairly narrow ion concentration range, and attempts to use one indicator to cover all experimental situations is likely to give inaccurate results. For example, the early fluorescent indicators for Ca ions were devised primarily to measure resting ion concentrations in small cells. The greatest accuracy for such measurements is obtained when the dissociation constant of the Ca-indicator complex is similar to the free Ca concentration, since the indicator fluorescence will be midway between its values at the concentration extremes. Unfortunately, this is not the best situation for measurement of transient concentration changes.

An indicator with a dissociation constant of a few hundred nM will be driven towards saturation by a Ca transient which locally elevates the free Ca concentration into the micromolar range, i.e. the slope of the Ca/fluorescence relationship will be progressively reduced as the Ca concentration increases. Although concentrations in this range can still be estimated if the Ca concentration is uniform throughout the measurement area, in practice this will not be the case. Instead, as the Ca diffuses away from the release sites, the total fluorescence signal will increase even though the average Ca concentration remains the same, because the reduction in fluorescence in the areas near the release sites is less than the increase in fluorescence in the areas further away. For accurate transient measurements, one should really use an indicator with a dissociation constant that is no lower than the highest concentration transient, but unfortunately this reduces the signal at resting ion levels.

Calibration

Although the calibration of fluorescent indicator recordings are, in general, easier than for photoproteins (which are highly non-linear) and for absorbance indicators (where absolute calibrations are difficult and interference effects from other ions tend to be greater), calibration can still be problematic. Ion concentrations can be estimated from fluorescence recordings by reference to *in vitro* calibration solutions, but such methods should be supplemented by measurements in which defined ion concentrations are imposed on the cell, to see if equivalent results are obtained (e.g. a known Ca concentration could be imposed by using an appropriate buffer solution outside the cell, and permeabilising the membrane with a Ca ionophore such as ionomycin, or perhaps an ion-sensitive electrode could be used). Unless such a verification can be made, calibrations should be taken only as a guide. This is a particularly important point, since calibration with reference to *in vitro* solutions is very straightforward, so it is always tempting to convert the results to concentrations without paying too much regard to possible errors.

For fluorescence measurements made at a single wavelength, the free ion concentration [I] is related to the fluorescence F by:

$$[I] = K_d(F-F_{min})/(F_{max}-F)$$

where F_{min} and F_{max} are respectively the fluorescence levels at zero and saturating ion concentrations, and K_d is the dissociation of the ion-indicator complex.

For fluorescence measurements made at a pair of wavelengths using ratiometric

indicators, the free ion concentration [I] is related to the fluorescence ratio R by the analogous equation:

$$[I] = K_d.S.(R-R_{min})/(R_{max}-R)$$

where S is a scaling factor given by the fluorescence at the denominator wavelength of R at zero ion concentration, divided by the fluorescence at a saturating ion concentration (see Grynkiewicz, Poenie and Tsien (1985) for derivation of this equation).

For single-wavelength indicators, F_{max} and F_{min} have to be estimated for each experiment because they depend on the indicator concentration, whereas for ratiometric measurements the parameters R_{min}, R_{max} and S (in theory) need to be measured only once on the experimental setup because by dividing the signals at two wavelengths the concentration terms in the equation are cancelled (although regular checks of these parameters are recommended). However, the values calculated from these equations need to be treated with some caution. Apart from the obvious risk that the K_d may be somewhat different in the cell from that in the calibration solution, there is also the possibility that the fluorescence properties of the indicator may be different in the cell, and such effects have been observed for several indicators (see above).

These equations also assume that the indicator is sufficiently selective that formation of complexes with other ions does not occur to any significant extent. Although this may be a questionable assumption, the fluorescent indicators generally perform quite well in this respect. This is particularly true for Ca indicators based on the BAPTA molecule, e.g. fura-2 and indo-1 (Grynkiewicz, Poenie and Tsien, 1985), which are more selective against Mg and protons than are the metallochromic Ca indicators, where competing effects from such ions pose a major calibration problem (Thomas, 1982, 1991). Where interference effects from other ions are significant, the simplest approach is to calibrate the indicator in a solution that contains appropriate concentrations of those ions. However, such calibrations will only apply so long as the concentrations of the interfering ions in the cell do not change significantly during the experimental procedures.

As discussed above, when the ester loading technique is used, additional problems occur. First, there is the risk that the indicator may not be completely de-esterified, giving rise to a variety of indicator species with different fluorescence and ion-binding characteristics, and thereby introducing further calibration errors. Second, de-esterification may occur in other cell compartments as well as in the cytosol (particularly in the endoplasmic reticulum), where the ion concentrations may be very different. In some cases this can cause entirely different results compared with those obtained by injection of free indicator.

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