Chapter 2
Measuring Ca^{2+} in Living Cells

Joseph Bruton, Arthur J. Cheng, and Håkan Westerblad

Abstract Measuring free Ca^{2+} concentration ($[Ca^{2+}]$) in the cytosol or organelles is routine in many fields of research. The availability of membrane permeant forms of indicators coupled with the relative ease of transfecting cell lines with biological Ca^{2+} sensors have led to the situation where cellular and subcellular $[Ca^{2+}]$ is examined by many non-specialists. In this chapter, we evaluate the most used Ca^{2+} indicators and highlight what their major advantages and disadvantages are. We stress the potential pitfalls of non-ratiometric techniques for measuring Ca^{2+} and the clear advantages of ratiometric methods. Likely improvements and new directions for Ca^{2+} measurement are discussed.

Keywords Ca^{2+} · Laser confocal microscopy · Fluorescence · Ratiometric · Non-ratiometric

Changes in the free Ca^{2+} concentration ($[Ca^{2+}]$) inside a cell can fulfil many different roles. Local changes in near membrane $[Ca^{2+}]$ can modify channels in the plasma membrane while changes in mitochondrial $[Ca^{2+}]$ can help to promote ATP production. Changes in nuclear $[Ca^{2+}]$ are critical for modulating gene replication and temporal aspects of these changes may provide valuable clues. One of the challenges in the field of Ca^{2+} signaling is to monitor the sites, amplitude and duration of free Ca^{2+} changes in response to physiological stimuli. Earlier researchers relied on a variety of methods, including atomic absorption and radioactive $^{45}Ca^{2+}$ to monitor Ca^{2+} in samples and Ca^{2+} movements across membranes and the likely underlying uptake and release mechanisms. Typically cell fragments were isolated by centrifugation and then Ca^{2+} uptake and storage capacity of isolated cellular organelles were examined. These methods were useful in the detection of relatively slow Ca^{2+} changes (seconds to minutes) but were unable to follow the short-term, transient Ca^{2+} movements induced by neural or

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hormonal stimuli. Nonetheless, they provided valuable information about Ca^{2+} in cells e.g. the majority of tissue Ca^{2+} exists as bound to the glycocalyx (extracellular cell coat, Borle [4]) and is essential for maintaining excitability of neurons and muscle cells. X-ray microanalysis or electron probe analysis was the most ambitious of these attempts looking at both cellular and subcellular changes in Ca^{2+} but even at its best, this technique reported only the result of a physiological stimulus and not what happened during the period of stimulation itself.

All of these earlier techniques looked at changes in total Ca^{2+} and could not distinguish between bound and free Ca^{2+}, but what is most relevant to physiologists is the free Ca^{2+} concentration. Free Ca^{2+} concentration in the cytosol is often written as $[Ca^{2+}]_i$, which can be confusing since the 'i' can be interpreted as meaning free or bound or both. In this review, $[Ca^{2+}]_i$ will be used to refer to the free cytosolic Ca^{2+} concentration. When muscle cells are electrically stimulated, free cytosolic $[Ca^{2+}]$ (i.e. $[Ca^{2+}]_i$) can increase more than tenfold in a few ms, whereas the intracellular $[Ca^{2+}]$ remains essentially constant. The transient increase in $[Ca^{2+}]_i$ is due to Ca^{2+} release from the sarcoplasmic reticulum (SR) into the cytosol and subsequent active removal from the cytosol. Thus, Ca^{2+} moves from one cellular compartment to another and back again and overall total intracellular $[Ca^{2+}]$ does not change.

Multiple bioluminescent and fluorescent Ca^{2+} indicators are now available to measure $[Ca^{2+}]$ in cells and subcellular regions. Published results focus often on amplitude and time course of the signal and gloss over the possible pitfalls of interpretation. Since many users are not experts and try to follow or modify methods described earlier, the likelihood of errors and misinterpretation of data has increased. Our focus in this chapter is to highlight what can and cannot be done with available Ca^{2+} indicators.

2.1 Earlier Attempts to Measure $[Ca^{2+}]$ Inside Cells

Measurements of $[Ca^{2+}]_i$ were rather complicated before the invention of the various fluorescent Ca^{2+} indicators that are commonplace today. An invaluable source of information about these methods is to be found here [3].

1. *Ca^{2+}-activated photoproteins*. In 1961, Osamu Shimomura spent a stressful summer mashing up the light organs distributed along the edge of the bell of many thousands of *Aequorea* jellyfishes trying to isolate and characterize what was responsible for the blue-green glow. These jellyfishes are pretty colorless in real life and do not spontaneously glow. However if they are poked or disturbed in the water, then a greenish bioluminescence is seen, localised only around the margins of the bell but not found anywhere else on the jellyfish's body. After many trials two proteins were isolated, the bioluminescent protein aequorin that glowed blue upon the addition of Ca^{2+} and the green fluorescent protein which in the living jellyfish produces green light because of resonant energy transfer

from aequorin. Shimomura was awarded the Nobel prize in Chemistry in 2008 for the green fluorescent protein discovery. Other bioluminescent proteins were subsequently isolated from other organisms (e.g. obelin, berovin) but none of them approached the versatility of aequorin either in their native form or with targeted mutations and thus they are hardly used today.

An advantage of aequorin is that as a bioluminescent molecule it does not require any external stimulating light and thus the background signal or noise is extremely low. On the other hand, the bioluminescence signal is quite small and measurement of the light emitted is not as easy as it is for other currently used fluorescent indicators. In practice, it is barely sensitive enough to following changes in resting $[Ca^{2+}]_i$. Even when aequorin is used to monitor changes in the high physiological range of $[Ca^{2+}]_i$ (0.5–10 μM) that are induced by electrical or chemical stimulation, there are difficulties in interpreting the light emission which increases as approximately the third power of $[Ca^{2+}]$. Translating aequorin light signal into actual values of $[Ca^{2+}]_i$ is complicated further by its consumption (i.e. the signal decreases over time) and since $[Ca^{2+}]_i$ differs within different regions of the cell (highest at release sites), the signal will be heavily dominated by the regions with the highest $[Ca^{2+}]_i$. The light emitted by aequorin in the presence of $[Ca^{2+}]_i$, will be influenced by Mg^{2+} and the ionic strength which can change markedly during intense stimulation. Moreover, it is sensitive to changes in pH especially below pH 7. It is useful to imagine the Ca^{2+}-activated photoproteins as being "precharged" and Ca^{2+} binding to an photoprotein molecule causes an energy-consuming reaction with emission of light that discharges the molecule. Each molecule emits light only once, which means that the light-emitting capacity declines over time but with experience and modelling, one can minimise this potentially confounding factor. In earlier days, the major problem with native photoproteins was getting them into a cell. In large cells this was achieved by microinjection which was not practical for smaller (< 20 μm) cells. Other loading techniques have been tried and of these, incubation combined with mild centrifugation seems to be the best. Once the sequence of aequorin was known, it became feasible to transfect cells and induce expression of recombinant aequorin. This works well with many cultured cells and in embryos but is problematic when one tries to induce expression in adult cells in culture or in a living animal. An advantage with this technique is that the aequorin gene can be modified and targeted to different cellular compartments (e.g. mitochondria or endoplasmic reticulum) and the Ca^{2+} binding properties of the proteins can be modified appropriately. Photoprotein-based methods to measure $[Ca^{2+}]$ in organelles are useful because in some situations it is not possible to introduce other fluorescent probes into a subcellular compartment [1].

2. *Metallochromic Ca^{2+} dyes*. Murexide was the first of these and arsenazo III and antipylazo III followed soon afterwards. With these indicators, the light absorbance of the molecule is monitored by a photomultiplier and when $[Ca^{2+}]$ increases, the light measured will decrease. The advantage of these dyes is that they are fast and therefore can detect rapid $[Ca^{2+}]_i$ transients. This is because

they display a relatively low Ca^{2+} affinity, which means that they readily can detect high $[Ca^{2+}]_i$ levels and show little Ca^{2+} buffering. However, there have some unwanted characteristics which include complex Ca^{2+}-binding properties, marked Mg^{2+} and pH sensitivity, and a tendency to bind to intracellular proteins. The metallochromic Ca^{2+} dyes do not easily enter intact cells, and therefore these dyes were usually microinjected. Today, with one exception, these dyes are seldom used by anyone except specialists looking at the kinetics and other properties of Ca^{2+} release in muscle cells. The exception is calcein a metallochromic indicator used since 1956 to look at calcium in minerals and salts. It is not sensitive to monitor resting $[Ca^{2+}]$ in unstimulated cells but has found a niche as a live live/dead cell indicator and looking at opening of the mitochondrial permeability transition pore.

3. *Ca^{2+}-selective microelectrodes*. Electrophysiological techniques were already used to probe channels in the plasma membrane and thus they could be readily adapted when suitable Ca^{2+} resin and ligands were produced by chemists. Double barrelled electrodes were adapted quite early on so that only one microelectrode impalement of the cell was necessary to measure both membrane potential and Ca^{2+} (the signal detected by the Ca^{2+} sensor includes both the membrane potential and the Ca^{2+} potential and thus, the membrane potential has to be subtracted). Ca^{2+}-selective electrodes are rather difficult to make since a special silane coat has to be applied to the glass first before the Ca^{2+}-selective ligand is loaded in the electrode [8]. Microelectrodes with tips less than 1 μm are used to minimise cell damage when the electrodes are inserted into cells. Ca^{2+}-selective microelectrodes have good selectivity for Ca^{2+} over other cations in the physiological range. They suffer from two drawbacks that have limited their use in Ca^{2+}-signalling. First they report the free $[Ca^{2+}]_i$ only in the vicinity of the microelectrode tip and second even under the best possible recording conditions, their response time is slow, on the order of seconds when changing between solutions containing different free $[Ca^{2+}]$. Thus, they are not able to follow the rapid $[Ca^{2+}]_i$ transients that occur in excitable cells such as muscle or neurons. Nonetheless, various groups have used them to report resting free $[Ca^{2+}]$ in both animal and plant cells as being 50 nM to 150 nM, slightly higher than was measured later with diffusible Ca^{2+} indicators and reflecting the fact that underneath and close to the plasma membrane, free $[Ca^{2+}]$ is higher than in the bulk of the cytosol.

2.2 Fluorescent Ca^{2+} Indicators

Many of the common Ca^{2+} indicators used today were derived from the Ca^{2+} chelator BAPTA developed by Roger Tsien and his co-workers [14]. The Ca^{2+} indicator molecule consists of two parts: the Ca^{2+}-binding cavity that changes its shape when Ca^{2+} binds to it and the scaffold part of the molecule giving

the fluorescence changes in response to Ca^{2+} binding to or being released from the cavity. These indicators have high selectivity for Ca^{2+} over Mg^{2+} and other common monovalent cations and are relatively unaffected by modest changes in H^+. When Ca^{2+} binds into the Ca^{2+}-binding cavity, there are large absorbance and fluorescence changes. It should be remembered that even with a low affinity for Mg^{2+} and H^+, Ca^{2+} indicators can be affected by these ions in experiments that are designed to induce metabolic exhaustion and thus a rise in free Mg^{2+} or large changes in pH. Much work has gone into developing different Ca^{2+}-binding properties and fluorescent tails that are optimised to work in defined ranges of $[Ca^{2+}]$ and with different types of detection systems.

Ca^{2+} indicators can be conveniently divided into two groups: single-wavelength non-ratiometric indicators and dual-wavelength ratiometric indicators. Indicators have absorption and emission spectra that have been well characterised in vitro and which apply in general to the behaviour of the molecules inside cells. Optimal excitation and emission wavelengths for individual indicators can generally be found in the papers where they were originally described and have been gathered here with additional details (https://www.thermofisher.com/se/en/home/life-science/cell-analysis/cell-viability-and-regulation/ion-indicators/calcium-indicators.html#crs).

Non-ratiometric indicators generally show very little fluorescence at low (<100 nM) $[Ca^{2+}]$ but show up to a hundred-fold increase in fluorescence when $[Ca^{2+}]$ increases maximally inside a cell so that the indicator becomes saturated with Ca^{2+}. The expectation that the light signal faithfully reflects $[Ca^{2+}]_i$, is probably true under ideal conditions. However, to be able to directly compare signals from different experiments the following requirements have to be fulfilled: (1) cells exposed to similar loading conditions will have similar concentrations of indicator; (2) indicators remain in the cytosol and do not leak or get pumped out of the cytosol; (3) cell volume remains constant and there is no change in cell thickness; (4) the cell does not move; (5) the indicator is not affected by repeated exposure to excitation light. Unfortunately all these requirements are almost never fulfilled and so data obtained with non-ratiometric indicators should be carefully assessed to avoid errors (see Fig. 2.1 and discussion below).

Ratiometric indicators have the advantage that the Ca^{2+}-free and Ca^{2+}-bound forms of the indicator have distinct peaks at different wavelengths. Thus, measurements can be made at the two separate peaks and combined into a ratio. The ratio is usually constructed so that the signal recorded at the wavelength where the fluorescence shows a maximum at high $[Ca^{2+}]$ is divided by the signal recorded at the wavelength showing its maximum at low $[Ca^{2+}]$. Between the two wavelength peaks, there is an isosbestic point where the fluorescence does not depend on $Ca^{2+}]$. In some cases (e.g. measuring quenching of a dye by Mn^{2+}) measurements are best made at this isosbestic wavelength. The classical ratiometric indicator fura-2 requires excitation at two wavelengths while the emitted fluorescent light is measured at one wavelength (~510 nm). The isosbestic point for fura-2 excitation is ~360 nm and with increasing $[Ca^{2+}]$, the emitted light increases at shorter wavelengths and decreases at longer wavelengths. The ratio with maximal dynamic

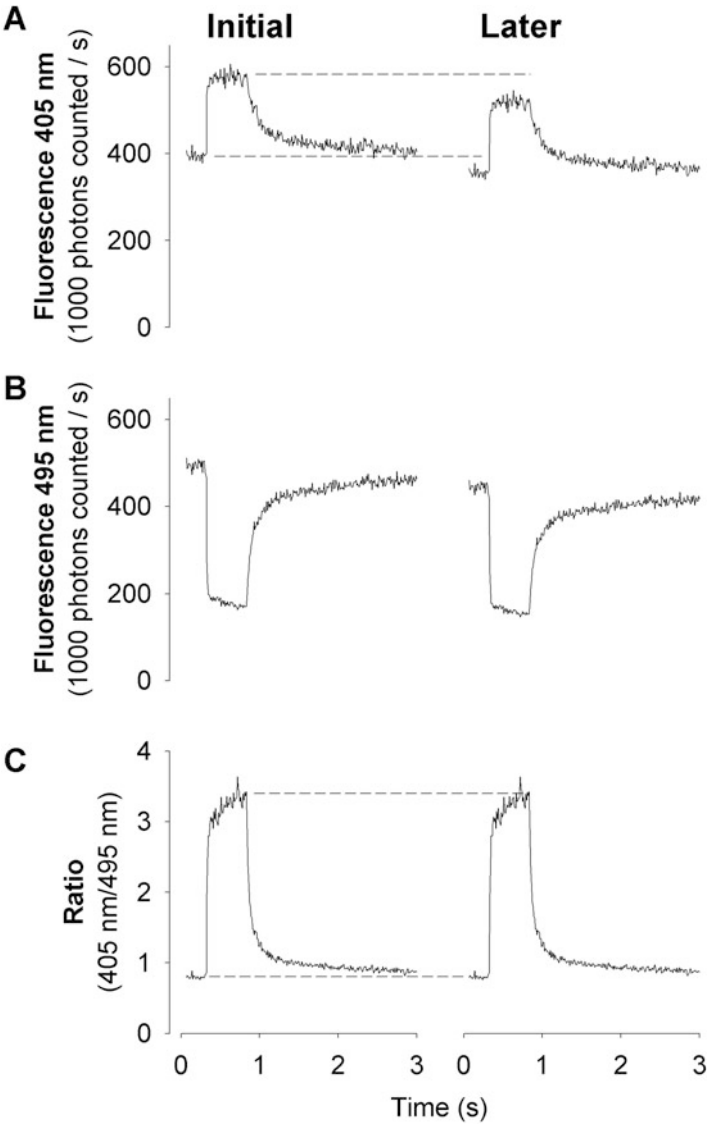

Fig. 2.1 Ratiometric indicators are best for experiments lasting hours. Indo-1 records obtained in a skeletal muscle cell stimulated to perform a tetanic contraction (70 Hz stimulation for 350 ms). Indo-1 was excited at 360 nm and the emitted light was measured simultaneously at 405 nm (**a**) and 495 nm (**b**) and the 405 nm/495 nm ratio was constructed (**c**). Over time, the ratio signal remained constant (dashed line in **c**) while the fluorescence intensity decreased for both the 405 nm (dashed line in **a**) and 495 nm signal. Note that the decline in the 405 nm trace seen in the right trace of the two shown in **a** is qualitatively similar to what would be seen if fluo-3 or another non-ratiometric indicator was used

range is then obtained by excitation below (~340 nm) divided by above (~380 nm) the isosbestic point. However, this requires continuous alteration between 340 nm and 380 nm excitation, which is technically troublesome, especially if rapid $[Ca^{2+}]_i$ transient are being measured. A simpler procedure is to measure the signal at the isosbestic point (360 nm) at regular intervals when constructing the ratio because the signal does not depend on $[Ca^{2+}]$. The preferred ratios will then be 340 nm/360 nm or 360 nm/380 nm, both of which will show an increase when $[Ca^{2+}]$ increases, albeit the ratio increase will not be as large as for the 340 nm/380 nm ratio. In our laboratory, we use the ratiometric dye indo-1 which requires excitation at only one wavelength and the emitted light be split into the $[Ca^{2+}]$-bound component (peaks about 400 nm) and the $[Ca^{2+}]$-free component (peaks about 475 nm).

The fundamental advantage of ratiometric over non-ratiometric indicators is exemplified in Fig. 2.1, which shows fluorescence records from a single skeletal muscle fiber at rest and during stimulation to produce a maximum contraction. Figure 2.1a shows the results as they would appear with a single wavelength indicator. As the experiments progressed, the fluorescent signal showed a general decline (probably representing pumping of the dye molecule out of the cell or transport by a non-specific anion transporter which can be blocked by probenecid or sulfinpyrazone), which might then be interpreted as a decrease in $[Ca^{2+}]_i$ both in the basal state and during contraction. However, the ratiometric indicator indo-1 was used in the experiment. In contrast to fura-2, this indicator is excited at one wavelength (~360 nm) and the emitted light is measured at two wavelengths (405 nm (increased signal with increasing $[Ca^{2+}]_i$) and 495 nm (decreased signal with increasing $[Ca^{2+}]_i$) in the depicted experiment). Figure 2.1b shows that there was a general decrease also in 495 nm signal as the experiment progressed. This means that there was no change in the 405 nm/495 nm ratio with time (Fig. 2.1c), which correctly reflects that there was no change in $[Ca^{2+}]_i$. The experimental traces in Fig. 2.1a show clearly that the signals from non-ratiometric indicators can result in completely erroneous conclusions if used without thinking. It should be noted that ratiometric indicators are not a cure for all problems. For instance, excessive UV light exposure can lead to qualitatively altered properties of the indicator (bleaching or inactivation), which cannot be corrected by ratioing [13].

2.3 Which Indicator Should One Use?

As outlined above, ratiometric indicators have clear advantages over non-ratiometric indicators and should be used whenever possible. Nowadays, visible-light laser scanning confocal microscopes are more common than any other Ca^{2+}-dedicated imaging systems meaning that a non-ratiometric indicator such as fluo-3/fluo-4 is often the first choice. Adding on a UV source to a microscope is reasonably straightforward and with suitable lens and filters, ratiometric indicators (i.e. fura-2 and indo-1 and their close relatives mag-fura-2 and mag-indo-1) could be used but this type of modification is rarely done.

In an ideal experiment, one would use an indicator which gives a fluorescence signal that shows large changes when $[Ca^{2+}]_i$ is changing and which is fast enough to follow the changes in $[Ca^{2+}]_i$ under study. However, the perfect indicator does not exist because some properties are difficult, or even impossible, to change. For instance, a Ca^{2+} indicator showing large changes in fluorescence with $[Ca^{2+}]_i$ changes in the low physiological range (~100 nM) is relatively slow and the opposite is also true. The relation between the intensity of the fluorescent signal (F) and $[Ca^{2+}]_i$ for a non-ratiometric indicator is given by the following equation (Eq. 2.1):

$$\left[Ca^{2+}\right]_i = K_d^* \ (F - F_{min}) / (F_{max} - F) , \tag{2.1}$$

where F_{min} and F_{max} mean the fluorescence intensity at virtually zero and saturating $[Ca^{2+}]_i$, respectively. K_d is the dissociation constant which in a plot of F against $[Ca^{2+}]_i$, will be the $[Ca^{2+}]$ where F is half-way between F_{min} and F_{max} and this is where the indicator displays its largest sensitivity. K_d is decided by an indicator's rates of Ca^{2+} binding (K_{on}) and dissociation (K_{off}), i.e. $K_d = K_{off}/K_{on}$. The on-rate constants of Ca^{2+}-binding are very fast and not that dissimilar whereas the rate that differs markedly between indicators is K_{off}. Accordingly, a slow indicator (low K_{off}) has a low K_d, which means that it is most sensitive at relatively low $[Ca^{2+}]_i$ and such indicators are therefore called high-affinity indicators. Conversely, a fast indicator has a high K_d and is referred to as a low-affinity indicator.

For ratiometric indicators, a slightly more complex equation describes the relation between fluorescence ratio (R) and $[Ca^{2+}]_i$ (Eq. 2.2):

$$\left[Ca^{2+}\right]_i = K_d^* \beta^* \ (R - R_{min}) / (R_{max} - R) , \tag{2.2}$$

where R_{min} and R_{max} is the fluorescence ratio at virtually zero and saturating $[Ca^{2+}]_i$, respectively. β is obtained by dividing the fluorescence intensity of the ratio's 2nd wavelength (denominator) acquired at virtually zero and saturating $[Ca^{2+}]_i$, respectively. Thus, the mid-point between R_{min} and R_{max} occurs at a $[Ca^{2+}]_i$ that equals $K_d * \beta$.

Figure 2.2 illustrates how the properties of two different Ca^{2+} indicators affect the change in fluorescence signal observed when $[Ca^{2+}]_i$ is changed in different concentration intervals. The comparison is between one high-affinity indicator, fura-2, and a low-affinity indicator, mag-fura-2. The name mag-fura-2 comes from the fact that it was designed to measure $[Mg^{2+}]$, but it has found its niche as a low-affinity Ca^{2+} indicator since $[Mg^{2+}]$ shows significant changes in the cytosol only when a cell is metabolically stressed by repetitive stimulation or exposed to poisons such as cyanide and its derivatives. $[Ca^{2+}]_i$ may vary dramatically between different physiological states. For instance, $[Ca^{2+}]_i$ peaks during contraction in a skeletal muscle cell may be up to 100-fold higher than resting $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ is therefore often expressed as pCa or the negative $\log[Ca^{2+}]_i$ (analogous to the concept of pH). In Fig. 2.2, the 340 nm/380 nm ratio is shown for both indicators and β is set to

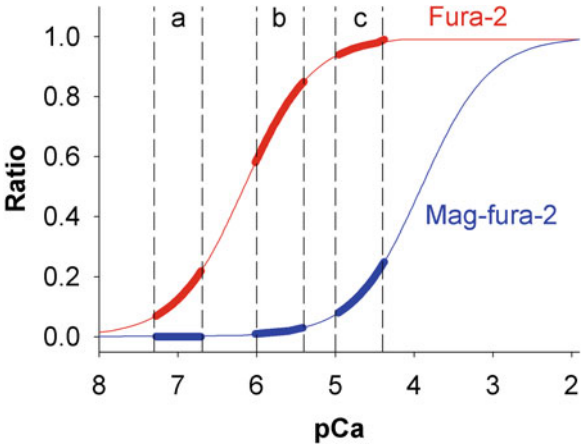

Fig. 2.2 High-affinity Ca^{2+}indicators are more sensitive to stable changes in $[Ca^{2+}]_i$in the normal physiological range. The relationships between $[Ca^{2+}]_i$ and fluorescence ratio (340 nm/380 nm excitation) are shown for the high-affinity indicator fura-2 and the low-affinity indicator mag-fura-2. $[Ca^{2+}]_i$ expressed as pCa ($-\log[Ca^{2+}]_i$) in order to cover a larger range of concentrations. The thick lines are used to emphasise the differences between the two indicators at different $[Ca^{2+}]$ (**a**) 50–200 nM; (**b**) 1–4 μM; (**c**) 10–40 μM

4. This means that the mid-point between R_{min} and R_{max} occurs at a $[Ca^{2+}]_i$ of 0.56 μM for fura-2 (K_d assumed to be 0.14 μM) and 100 μM for mag-fura-2 (K_d assumed to be 25 μM). The interval (a) in Fig. 2.2 shows the change in ratio signal obtained when $[Ca^{2+}]_i$ is changed in the range of normal resting values, 50–200 nM. Here the fura-2 ratio signal shows a substantial increase, whereas mag-fura-2 ratio signal changes hardly at all. Thus, fura-2 can readily detect changes in basal $[Ca^{2+}]_i$, whereas mag-fura-2 is useless. The interval (b) in Fig. 2.2 (1–4 μM) would reflect $[Ca^{2+}]_i$ in cells that are activated. Again fura-2 is a rather sensitive indicator in this interval, whereas mag-fura-2 shows little change in the ratio signal. Finally the area marked (c) reflects $[Ca^{2+}]_i$ (10–40 μM) in a cell stimulated to maximal activation. In this case, fura-2 is saturated and changes little in the face of large concentration changes, whereas mag-fura-2 is clearly able to report changes in $[Ca^{2+}]_i$.

As a rule of thumb, all buffers are useful for detecting changes in an interval between about tenfold below and tenfold above the mid-point. Thus inside a cell where free $[Ca^{2+}]$ varies between 50 nM and 2–3 μM a suitable Ca^{2+} indicator which readily detects $[Ca^{2+}]_i$, at rest and during activation will have a K_d of 200–300 nM. This is also the $[Ca^{2+}]_i$ interval where Ca^{2+} most easily binds to the indicator, which has the potential to cause buffering problems. The noise in the detected fluorescent light signal decreases with increasing emitted light intensity. From this perspective it would be advantageous to have a large concentration of fluorescent indicator in a cell. However, a high concentration of indicator with a K_d in the physiological $[Ca^{2+}]_i$ range will buffer $[Ca^{2+}]_i$ markedly as illustrated in Fig. 2.3. When a relatively low concentration of indicator is present in the cell

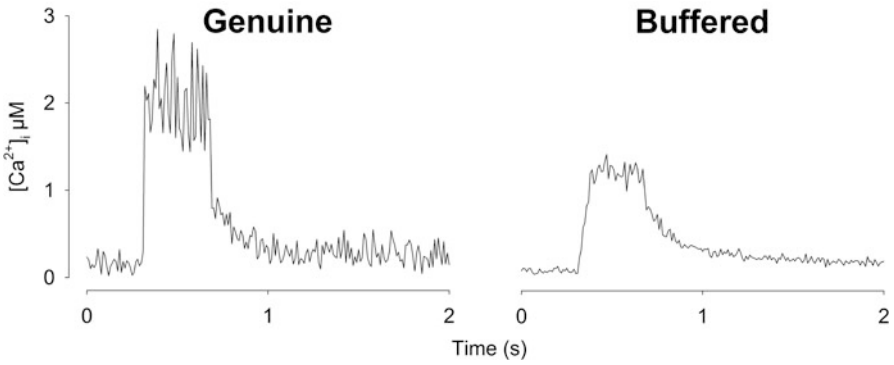

Fig. 2.3 Excessive cytosolic loading of Ca^{2+} indicator distorts $[Ca^{2+}]_i$ transients. Typical records from the same skeletal muscle cell illustrate the real $[Ca^{2+}]_i$ response to 70 Hz tetanic stimulation (left trace) and the response as it looked after further injections of the Ca^{2+} indicator, indo-1, that caused buffering of the $[Ca^{2+}]_i$ transient (right trace). Note the reduced noise, the reduced amplitude and the slower rate of rise and decay in the buffered Ca^{2+} transient compared to the original record

("Genuine") a rapid and relatively large change in $[Ca^{2+}]_i$ is recorded but the signal contains some irregular fluctuations (noise). A markedly higher concentration of indicator ("Buffered") gives a far less noisy signal but the time course of the rise and fall of $[Ca^{2+}]_i$ is slowed and the amplitude of the change is less. Thus, with high-affinity Ca^{2+} indicators there is a delicate balance between introducing a sufficiently high indicator concentration to obtain records with an acceptable noise level and having so much indicator that cytosolic Ca^{2+} is markedly buffered, which leads to distorted $[Ca^{2+}]_i$ signals as well as altered cell signalling or function.

Figure 2.2 shows that a high-affinity Ca^{2+} indicator is better than a low-affinity indicator at monitoring changes in $[Ca^{2+}]_i$ in the normal physiological range. However, the diagram in Fig. 2.2 refers to stable or slowly changing $[Ca^{2+}]_i$. As discussed above, a trade-off of high Ca^{2+} sensitivity is that the indicator may be too slow to follow rapid changes in $[Ca^{2+}]_i$. In Fig. 2.4 this is illustrated for $[Ca^{2+}]_i$ transients in a skeletal muscle cell, the same would be true for any other excitable cell. The $[Ca^{2+}]_i$ transient resulting from a single stimulation pulse lasts for ~10 ms. Figure 2.4a shows such a $[Ca^{2+}]_i$ transient as recorded with the high-affinity indicator indo-1. However, the indicator is not fast enough to accurately follow the rapid changes in $[Ca^{2+}]_i$ and the recorded transient is too slow and the amplitude too low. In Fig. 2.4b the signal has been kinetically corrected [15] to take account of the properties of indo-1 and the $[Ca^{2+}]_i$ transient now better represents the true situation. While a low-affinity Ca^{2+} indicator could follow $[Ca^{2+}]_i$ transients more accurately and would therefore be preferable in experiments where rapid $[Ca^{2+}]_i$ changes are being studied, there is the drawback that the change in fluorescent signal is going to be small and hence difficult to measure. Figure 2.4c shows $[Ca^{2+}]_i$ as recorded by indo-1 during tetanic stimulation (70 Hz, 350 ms duration) of the

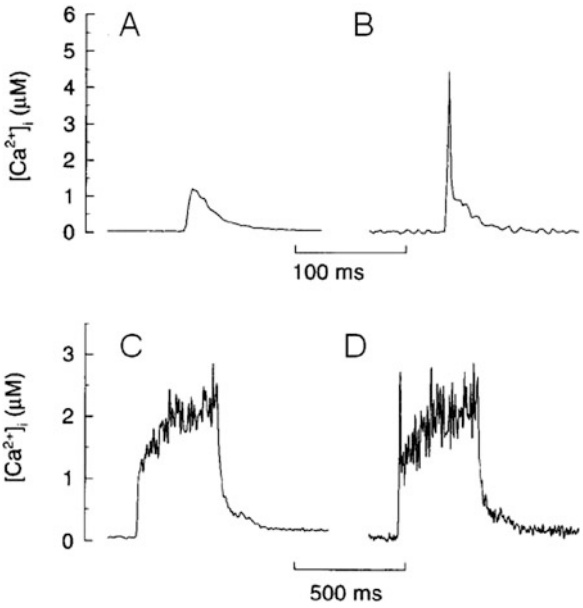

Fig. 2.4 Fast low-affinity Ca^{2+}indicators or kinetic correction of high-affinity Ca^{2+}indicator records are required to accurately portray rapid $[Ca^{2+}]_i$transients. $[Ca^{2+}]_i$ records measured with indo-1 in a skeletal muscle cell in response to a single stimulation pulse (**a**) and a tetanus (**c**). This high-affinity indicator is too slow to accurately follow the most rapid changes in $[Ca^{2+}]_i$. Kinetic correction reveals a faster and larger $[Ca^{2+}]_i$ transient with the single stimulation pulse (**b**) and a $[Ca^{2+}]_i$ spike at start of the tetanus (**d**). (Figure adapted from Westerblad and Allen [15])

muscle cell; in Fig. 2.4d the record is corrected for the slow response of indo-1. It can be seen that the initial "spike" of $[Ca^{2+}]_i$ is missed without correction, but otherwise the records are rather similar. To sum up, Fig. 2.4 thus illustrates that problems with slow, high-affinity Ca^{2+} indicators are substantial when recording rapid $[Ca^{2+}]_i$ transients but much less so during more prolonged $[Ca^{2+}]_i$ changes. Thus, again there is a delicate balance between being able to measure large and rapid $[Ca^{2+}]_i$ changes (low-affinity indicators are preferable) and measure small prolonged changes (high-affinity indicators are better).

The signals recorded by the PMT or CCD are always transferred and stored on a computer and this means that the sampling rate can be high as one wishes. Sampling theorems are available which can be used empirically to decide what the optimal sampling rate is. As a general rule, we use a sampling rate at least tenfold faster (100 Hz to 1 kHz) than the expected fastest speed of $[Ca^{2+}]_i$ transients under study. It is worth remembering that using a high sampling rate means that less light signal is integrated for each time point and hence the noise level is higher with fast than with slow sampling. On the other hand, rapid or small $[Ca^{2+}]_i$ transients might be missed or distorted with a low sampling rate.

2.3.1 How Easy Is It to Get Indicators into Cells?

Indicators are charged molecules and do not easily pass lipid membranes. While many cells display endocytotic behaviour, we consider that the amount of indicator that can enter the cell by endocytosis during a couple of hours will be small and unlikely to be enough to make reproducible and meaningful measurements. Ca^{2+}-indicator can be introduced into cells by pressure injection or by electrophoreses. Electroporation of the cell membrane using very brief, high voltage pulses opens transient small pores in the cell membrane through which indicator molecules pass. All of these techniques require specialised equipment and some skill, but they maximise the likelihood that the indicator will be found only in the cytosol and not move into sub-cellular compartments, such as the mitochondria or sarco-endoplasmic reticulum.

Fortunately, there is a much easier method for introducing fluorescent indicators into the interior of single cells or tissue. The principle behind the method is that lipophilic groups (acetoxymethyl or acetate ester (AM) groups) are added to the charged indicator molecule. In this way the charges are hidden and the indicator complex becomes lipophilic and hence membrane-permeant. Once the complex has entered into the cytosol, cytoplasmic esterases gradually cut off the lipophilic groups and the free indicator molecule is then trapped in the cytosol and ready to detect $[Ca^{2+}]_i$. This simple method of loading fluorescent indicators into cells gives many a chance to investigate the regulation of $[Ca^{2+}]$ in their favourite cell. The lipophilic AM-indicator complex is typically dissolved in a mixture of dimethylsulfoxide (usually written as DMSO) and the detergent Pluronic to disperse the indicator molecules and aid cell loading. Typically, cells are exposed to the indicator (1–10 μM) for 10–30 min. After the loading period is finished, the cells are washed to remove residual extracellular AM-indicator and left for a further 30 minutes to ensure that all lipophilic groups have been cleaved off by cytoplasmic esterases allowing the indicator molecule to interact with Ca^{2+}. We have successfully used this basic loading protocol to detect electrically- or chemical-induced transient changes in $[Ca^{2+}]_i$ in myoblasts, myotubes and muscle fibres [12], pancreatic beta-cells [5], hippocampal neurons [10] and cardiomyocytes [11].

Loading of the lipophilic AM-indicator complex is not without problems. The quantity loaded into cells cannot be directly controlled. This leads to the risk of excessive loading and resultant buffering of $[Ca^{2+}]_i$, which affects Ca^{2+} homeostasis inside the cell and gives erroneous estimates of changes in $[Ca^{2+}]_i$ amplitude and time course as well as affecting Ca^{2+}-dependent cellular signalling. An additional problem with AM-indicators is that they may pass across intracellular membranes into organelles and report changes in $[Ca^{2+}]$ in this compartment in addition to changes in the cytosol. Our experience is that these problems seem to be minimised if cells are loaded at room temperature rather than at the higher physiological temperature of mammals. Unfortunately there is no single set of conditions that produces optimal loading of all cells and procedures needs to be optimised for each new cell type. For example, in our hands, indo-1 AM does not

load into mouse cardiac myocytes but does load into rat cardiac myocytes. It should be noted that in tissues or densely coated cultured cells, indicator molecules can be trapped and cleaved by extracellular esterases to produce an indicator that reports extracellular [Ca^{2+}] and confounds the intracellular measurements [9].

For quantitative measurement within the cytosol of rapid or repeated transient [Ca^{2+}] in any intact neural or muscle cell, indo-1 is our first choice of fluorescent indicator. For slower changes lasting seconds or minutes, either indo-1 or fura-2 would be adequate. If one is interested only in the effect of a drug or other intervention and not the absolute numbers, then one could easily turn to fluo-3/fluo-4 as first choice indicators. For looking at intracellular organelles, the fluorescent indicator rhod-2 has been widely used to monitor changes in mitochondrial [Ca^{2+}] in neurones and muscles during and after stimulation by us and others. The low-affinity calcium indicator (K_{Ca} 90 μM) fluo-5 N has been used to monitor SR [Ca^{2+}] during repeated tetanic contractions. [Ca^{2+}] measurements can also be attempted using compartment-specific aequorin chimeras and other genetically engineered proteins [1, 7].

2.4 Equipment Overview

Typically, one uses the instruments that are available rather than those that are optimal for the task of measuring changes in [Ca^{2+}] inside a cell. The minimum needed to detect the fluorescence emitted from cells loaded with an indicator are a microscope with a light source to locate the cells and to excite the indicator, a detection device that is typically one or more photomultiplier tubes or a CCD camera and some recording or storage device. A simpler fluorometer-based system can be used if one is working with cell suspensions and is not interested in the response of individual cells. Filters are inserted into the light path to limit the wavelength and intensity of the light that excites the indicator and also to limit the wavelengths of the emitted light measured by the light detectors. The signals from the light detectors are generally digitised and stored on a computer. Newly purchased equipment dedicated to Ca^{2+} measurements is supplied with software controlling the various parameters related both to excitation wavelength and to detection of the emitted light that is more than capable of recording and performing a fast on-line analysis of signals.

The most important but often neglected part of the whole acquisition system is the light path and especially the objective lens. The lens is what allows one to magnify and focus on the cell or tissues. While magnification is important to see the sample, what is equally or more important is the ability of the lens to pass light of the appropriate wavelength and resolve fine specimen detail. The light collection effectiveness is described by the numerical aperture (N.A.) written on the lens casing. In general, one should have the lens with the highest N.A. possible (a more detailed description can be found here: http://micro.magnet.fsu.edu/primer/anatomy/numaperture.html). Lenses that are optimised to work with ultraviolet (UV) light are not optimal for visible light and vice versa. Lenses are

exposed to the dust and moisture in the working environment unlike most of the other elements of the system, which are encased in protective housing. Even if an acquisition system is handled carefully, the lens is liable to become dirty from the particles floating in the air. If the lens requires oil or water for its proper operation, the combination of liquid and dust can lead rather quickly to the formation of a film coating the lens surface and the light path deteriorates. We use a superfusion system routinely in our experiments and over the years we have had a variety of problems ranging from leaks in aged tubing, overflow of liquid out of the recording chamber resulting in fluid on and inside the lens leading to a rapid deterioration of the signal. If not spotted quickly, this can lead to salt deposits on the lens or, in the worst case scenario, fluid entering the lens casing with a salt coating both outside and inside the lens. Problems of this kind are easily recognised as increased noise in the fluorescence signal and in the worse cases inability to focus on the cells or tissue. It should be routine to check the lens before and at the end of an experiment and to clean the lens with lens paper and an air spray before and after experiments or immediately one sees that solution has dripped on to the lens. If solution has dried and formed salt crystals on the lens, we use distilled water to rinse the salts away and ethanol is used finally to clear off residual water.

Nowadays, the most common types of detection set-ups are epifluorescence microscopy and scanning confocal microscopy. In epifluorescence microscopy, the whole sample consisting of a single cell or group of cells loaded with an indicator is excited by light of the appropriate wavelength and the photons emitted from the indicator are collected both from the sample section in focus (typically 0.3 μm with an objective lens with a high numerical aperture of 1.3) and also from above and below this plane of focus. Emitted light travels to one or more photomultiplier tubes or a CCD camera. Epifluorescent microscopy is used most commonly with ratiometric dyes such as indo-1 or fura-2 that are excited with light in the UV region. This type of set-up is ideal for measuring changes in $[Ca^{2+}]_i$ in virtually any cell type over extended periods of time while using mechanical, electrical or chemical stimulation. The area of interest can be limited to a single cell or data can be collected from a larger number of cells. While this method allows one to measure from the total volume of the cell, it is difficult (or with photomultiplier tubes basically impossible) to focus in on discrete areas of the cell and visualise events such as the entry of extracellular Ca^{2+} through surface membrane Ca^{2+} channels. However, when combined with special indicators, one can measure $[Ca^{2+}]$ changes in discrete organelles. For example, rhod-2 is a Ca^{2+}-indicator that loads preferentially into the mitochondria and indo-5 N has been used for measurements of $[Ca^{2+}]$ in the sarcoplasmic reticulum. Several groups including us have measured changes in $[Ca^{2+}]$ in the vicinity of the plasma membrane rather than in the bulk of the cytoplasm using an indicator moiety conjugated to fatty acid chains called FIP-18 which preferentially anchors into the surface membrane and measures $[Ca^{2+}]$ nearby (e.g. https://www.scbt.com/scbt/sv/product/ffp-18-am).

Confocal microscopy uses much the same hardware and software as that used in epifluorescence microscopy with two important additions: a laser acting as a point light source that excites the indicator and an adjustable diaphragm or pinhole in the

emission pathway that when opened to its optimal size lets through light only from the focal plane, i.e. reducing light collection from cell regions outside the plane of focus. The fundamental advantage of the confocal microscope is that one can limit the focus to a very narrow section and thus measure discrete and rapid events such as localised release/entry of Ca^{2+} into the cytoplasm. While most confocal microscopes use lasers as light sources, this is not essential and the type of light source was not specified in the original patent (http://web.media.mit.edu/~minsky/papers/ConfocalMemoir.html).

Laser confocal microscopes come in three basic designs. These are (i) single photon laser scanning, (ii) the Nipkow or spinning disk, and (iii) two-photon versions.

(i) The single photon laser scanning confocal microscope is found in almost every biological/physiological institution. Most popular are those supplied by the major microscope manufacturers but nowadays for those who are technically proficient, it is possible to buy a confocal kit from the big optical suppliers (e.g. Thorlabs) and retrofit it to an existing microscope setup. In most systems, solid state lasers which have very precise and stable light emission and will work for many years have replaced Kr/Ar gas lasers. Physicists explain excitation of an indicator molecule as occurring when a single photon of the appropriate wavelength hits an indicator molecule and transiently lifts it from its ground state to a higher energy state. It remains in this higher energy state briefly (picoseconds) and then decays back to its original ground state by emitting a new photon with a longer wavelength than the original incident photon. An image of the sample is built up by moving a laser beam rapidly from one point to an adjacent point (pixel to pixel, typically dwelling a few to tens of μs on each pixel) along a horizontal line by means of a pair of mirrors (galvanometer-controlled or resonant-oscillating). A two dimensional image is built up by moving the laser beam vertically to a new line with a second pair of mirrors. The scanning and vertical movements are repeated until a full frame is obtained. This obviously takes a finite period of time and does not give an instantaneous view of what is happening in the cell. One can increase the scanning speed and obtain a full frame two to three times faster by reducing the "dwell time", i.e. the time for which the laser illuminates each pixel. The disadvantage of doing this is that the signal to noise ratio is reduced, which limits the ability to monitor small, spatially restricted changes in the fluorescence signal. If temporal resolution of a Ca^{2+} event in the cell is critical, the best approach is to abandon the two dimensional image acquisition approach and use the line scan mode instead. In this configuration, the laser beam scans the same line sequentially for a period of time. Line scans can be performed at over 1 kHz which is sufficiently fast to resolve even the most rapid change in local Ca^{2+} in a cell. The trade-off for the increased speed of data acquisition with line scanning is that only a single plane in a portion of the cell or tissue can be monitored. The line scan mode is extremely useful if one is trying to identify and characterise localised transient releases of Ca^{2+} from the

sarcoplasmic reticulum in muscle or trying to localise the sites of Ca^{2+} entry in a neuron. Conversely, the full frame ("x-y mode") is best if one is trying to see what happens in the whole cell in response to a stimulus.

(ii) Spinning disk laser confocal microscopes use a spinning disk (rotating at several thousand revolutions per minute) with multiple pinholes (> 1000) through which parallel light beams pass. These beams excite the fluorescent indicator in the cell and the emitted light returns through a second collector disk with a matching pattern of microlenses to the detection device, which is normally a very sensitive CCD camera operated at low temperatures to minimise noise. The current generation of spinning disk confocals can easily acquire images at rates of up to 50 frames per second, which makes them suitable for visualizing temporal and spatial [Ca^{2+}] changes in in a whole cell or cells rather than just a restricted line or set of lines using the line scan mode of a scanning confocal microscope. High frame rates generate large volumes of data but supplied software or ImageJ (download free from NIH) are sophisticated enough to select and analyze regions of interest only while masking data from uninteresting areas. The limited lack of popularity of these confocal microscopes may in part be due to the trade-off between spatial resolution and speed, i.e. greater spatial resolution generally requires a slower frame rate of acquisition and in part to the amount of incident light required that at best causes bleaching of the Ca^{2+} indicator only and at worst results cell damage and death.

(iii) The two- or multi-photon confocal microscopes overcome problems occurring when deeper parts of cells or tissues are being studied. Every microscope can be fitted with a motorised drive that accurately moves the plane of focus up or down in steps smaller than 1 μm. Thus, one can theoretically build up a three dimensional confocal image of a cell or tissue and check for possible hotspots or non-homogeneous change in [Ca^{2+}] throughout a cell, tissue slice or cell culture in response to a stimulus. However, with a simple laser confocal scanning microscope, image quality deteriorates as one penetrates deeper into a cell or tissue. This impaired performance is due to the fact that a laser beam is a stream of photons that will excite any indicator molecule it meets as it travels to the plane of focus. Thus, a lens will receive photons not just from the plane of focus but also some photons that have been deflected into the light collection path following collision with proteins. As the distance from the region of interest to the lens increases, some photons from the focal plane of interest will be lost and photons from uninteresting regions will be collected.

Two-photon confocal microscopes minimises this problem by delivering the longer wavelength pulses required to excite indicator molecules only to a very confined region. The longer wavelength improves penetration depth into tissue which is especially important when looking at the behaviour of nerves in the brain or secretory cells in isolated parotid or pancreatic ducts. The beauty of the two photon technique is that excitation of an indicator molecule can only occur if two photons each with twice the wavelength and half of the energy of a single photon

hit an indicator molecule. Indicator molecules hit by only one photon will not be excited. Longer wavelength light is less likely to cause damage to the cells. In a two-photon laser, the photons are sent out in femtosecond bursts. At the focal point, there is a high density of photons and the probability of two photons colliding with an indicator molecule is high. The major factor limiting more widespread usage of two-photon microscopy is the cost of the pulsed lasers themselves.

A final caution about experiments with lasers and intense light should be made. Children are routinely reminded to sunbathe in moderation and minimise prolonged exposure to ultraviolet light and avoid skin damage. The experience of seeing a cell start to bleb and die as one struggles to obtain the best record of $[Ca^{2+}]$ transients highlights the fact that light energy is dangerous to cells. One should be aware that the energy that each photon of light contains may impact on the measurements being made and should try to limit the intensity of the light to the minimum possible. An additional problem is that intense light may produce photodegradation or photobleaching of Ca^{2+} indicators whereby the indicator is converted into a fluorescent but Ca^{2+} insensitive form that results in false measurements of resting and transient changes in $[Ca^{2+}]_i$ [13]. Again, the problem can be avoided by minimising the intensity and duration of light exposure.

2.5 Calibration of the Fluorescent Signal

Some kind of calibration is usually attempted in order to translate fluorescence signals into $[Ca^{2+}]_i$. Before any calibration is attempted, it is important to recognise that there is always some background signal in fluorescence systems, arising from the detectors themselves and because of imperfect filters and leakage of the excitation light to the detectors. Moreover, each tissue or cell will have an intrinsic or auto-fluorescence. The autofluorescence arises predominantly from proteins containing the amino acids tyrosine, tryptophan, and phenylalanine. The amount of background and intrinsic fluorescence depends on the excitation and emission wavelengths being used. It is necessary to measure the background and intrinsic fluorescence in a sample before loading the Ca^{2+} indicator and to subtract this value from all subsequent measurements. Failure to do this can have dramatic effects on the translation of the indicator signals into $[Ca^{2+}]_i$. Complete and accurate calibrations are generally tiresome or even impossible to perform on a single cell and some simplifications are usually made. This has led to an increased tendency to completely ignore calibrations and take the viewpoint that the fluorescence light intensity (F, non-ratiometric indicators) or ratio (R, ratiometric indicators) of Ca^{2+} indicators is linearly related to $[Ca^{2+}]_i$, which clearly is a severe oversimplification (e.g. see Fig. 2.2). Numerous papers erroneously state that $[Ca^{2+}]_i$ increased/decreased by x%, whereas what actually occurred was an increase/decrease in fluorescence intensity or ratio of x%, which can represent

markedly different changes in $[Ca^{2+}]_i$. For instance, a minimal (<1%) change in fluorescence signal measured in a resting cell with a low-affinity indicator may represent a several-fold change in $[Ca^{2+}]_i$ (see Fig. 2.2). Similarly, a major increase in $[Ca^{2+}]_i$ may result in only a small increase in the fluorescence signal of a high-affinity indicator because the indicator was almost saturated with Ca^{2+} already before the increase.

Ca^{2+} indicators are affected by the surrounding protein and ionic environment and hence their properties inside a cell and in a test-tube will be markedly different. The relationship between fluorescence signals and $[Ca^{2+}]_i$ will also depend on the experimental setup. This means that all parameters in Eqs. 2.1 and 2.2 required to translate fluorescence signals into $[Ca^{2+}]_i$ should be established in the cell(s) using the same conditions and equipment as for the real experiments. This is of course easier said than done and some shortcuts are usually taken. In principle, the intracellular calibration is based on clamping $[Ca^{2+}]_i$ to a known value, without severe alterations of the cytosolic milieu, and then measure the fluorescence signal. The most important points to measure are at low/minimum $[Ca^{2+}]_i$, using EGTA or BAPTA to chelate Ca^{2+} to obtain F_{min} or R_{min}, and at saturating $[Ca^{2+}]_i$, to establish F_{max} or R_{max}. For ratiometric indicators, β is also obtained if R_{min} and R_{max} can be established without any major general decrease in fluorescence intensity. In addition, establishing K_d requires some intermediate $[Ca^{2+}]_i$. The reason why F_{min} or R_{min} and F_{max} or R_{max} are most important is because they set the limits between which the fluorescence signal can vary. Errors in measuring these parameters result in nonlinear errors when fluorescence signals are translated into $[Ca^{2+}]_i$. Erroneous estimates of F_{min} or R_{min} has the largest impact on the assessment of resting $[Ca^{2+}]_i$, whereas errors in F_{max} or R_{max} have the largest effects at high $[Ca^{2+}]_i$. On the other hand, K_d and β act as scaling factors and errors in these simply make the absolute changes in $[Ca^{2+}]_i$ smaller or larger, whereas relative changes during the course of an experiments are not affected.

Numerous methods have been used to perform a cytosolic calibration of $[Ca^{2+}]_i$. Most of these are based on introducing a strongly buffered solution with a set $[Ca^{2+}]$ to the cytosol. The solution can be introduced with methods similar to those described above for the introduction of the fluorescent indicator. An easy way of getting Ca^{2+} into cells is to add ionophores such as ionomycin or A23187 or even beta-escin to make the cell membrane leaky.

2.6 What Can We Hope for Now?

There have been marked improvements in the level of resolution. It was known and accepted for more than a century that separation of two objects closer than 250 nm in the horizontal plane was not possible with a standard single lens and

light source. However, the use of two opposing and matched objective lenses and a complementary approach that relies on the photochemical properties of the indicators have led to at least a threefold improvement in both axial and horizontal resolution. While these technical improvements are still expensive to implement and are not yet generally available as ready to use equipment packages, it is likely that super-resolution fluorescence microscopy techniques will be used to image Ca^{2+} fluxes through groups of ion channels in the future (the clearest non-technical introduction is given in Hell [6]).

In recent years, different groups have further developed genetically encoded Ca^{2+} indicators (GECI's) and focussed on improving different aspects of their performance. The key to these developments was the recognition that the green fluorescent protein (GFP) found in jellyfish could be modified relatively easily to produce variants in various colours.

Green fluorescent protein GECI can be split into two broad groups. The first group are proteins that consist of a fusion of circularly permutated green fluorescent protein (GFP) or red fluorescent protein, a Ca^{2+}-binding protein (usually calmodulin or troponin C) and M13 (a short Ca^{2+}-CaM-binding peptide derived from from myosin light chain kinase that acts as a spacer). This shows weak fluorescence in the absence of Ca^{2+}. When Ca^{2+} binds there is change in its conformation and the protein construct now fluoresces brightly. The second group consists of the cameleons that rely on resonance energy transfer (FRET) to signal changes in $[Ca^{2+}]$. FRET works only if the two molecules making up the FRET pair are very close together (< 10 nm). Cameleons are a fusion of calmodulin binding Ca^{2+} to M13 and flanked on one side by a blue-shifted GFP and on the other side by a longer wavelength shifted GFP. When Ca^{2+} binds to calmodulin, the distance between the GFP molecules is altered and FRET efficiency increases. The cameleons are inherently ratiometric allowing one in theory at least to translate the FRET pair ratio into real $[Ca^{2+}]$. Since these complex proteins are genetically encoded, they have been targeted successfully to subcellular compartments. Interference from native forms of the Ca^{2+}-binding protein has been reduced through selective mutations. Their dynamic range has improved markedly but the maximum change of about 50% on average is markedly less than the classical fluorescent indicators such as indo-1 and fluo-3.

The 22 kDa bioluminescent protein aequorin and its prosthetic protein (coelenterazine) that is oxidised and released when Ca^{2+} binds have been massively re-engineered to optimise the properties of the photoprotein for monitoring of Ca^{2+} at different sites inside a cell [2]. Despite all the improvements, the inherent limitations of low light emission (one photon per aequorin versus hundreds of photons for other indicator molecules) and its consumption continue to make recording and interpretation of experiments difficult. It is difficult to see further improvements in this area.

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