A Simple Method for High Temporal Resolution Calcium Imaging with Dual Excitation Dyes

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ABSTRACT Calcium-sensitive dual excitation dyes, such as fura-2, are now widely used to measure the free calcium concentration ($[Ca^{2+}]$) in living cells. Preferentially, $[Ca^{2+}]$ is calculated in a ratiometric manner, but if calcium images need to be acquired at high temporal resolution, a potential drawback of ratiometry is that it requires equally fast switching of the excitation light between two wavelengths. To circumvent continuous excitation switching, some investigators have devised methods for calculating $[Ca^{2+}]$ from single-wavelength measurements combined with the acquisition of a single ratiometric pair of fluorescence images at the start of the recording. These methods, however, are based on the assumption that the concentration of the dye does not change during the experiment, a condition that is often not fulfilled. We describe here a method of single-wavelength calcium imaging, in which the dye concentration is estimated from ratiometric fluorescence image pairs acquired at regular intervals during the recording period, that furthermore includes a correction for the changing dye concentration in the calculation of $[Ca^{2+}]$.

INTRODUCTION

The measurement of the free calcium concentration ($[Ca^{2+}]$) in the cytoplasm of living cells with video microscopy and calcium-sensitive fluorescent dyes is now a well-established technique (Moore et al., 1990; Roe et al., 1990; Tsien and Harootunian, 1990; Moreton, 1994). For $[Ca^{2+}]$ imaging studies, dual excitation ratiometric calcium dyes, such as fura-2, have the advantage that these dyes only require a single intensified camera at the detection site. With a single camera, the need for pixel alignment is avoided and equipment costs are lowered. However, to obtain images of $[Ca^{2+}]$ with a high temporal resolution, ratiometric dyes require a rapid, steplike change in the excitation wavelength. This change in excitation illumination is commonly achieved either with a rotating filter wheel containing the appropriate optical bandpass filters or with an optical switch that alternates between two light sources of different wavelengths generated by stationary filters or monochromators. An additional requirement is that the recording camera must be capable of responding quickly to, and be synchronized with, the changing excitation. Although low-lag intensified CCD cameras and video rate excitation switching devices are available, this equipment is frequently expensive. In addition, if images need to be acquired at rates above the standard video rate (NTSC 30 Hz, CCIR 25 Hz), synchronized excitation switching can become difficult to achieve.

To eliminate the need for rapid and repetitive switching between two excitation wavelengths, other investigators have reported an alternative approach to determining $[Ca^{2+}]$ from single-wavelength measurements (Monck et al., 1988; Vranesic and Knöpfel, 1991; Silver et al., 1992). This approach is based on the recording of a single fluorescence ratio pair at the start of the experiment, from which the initial $[Ca^{2+}]$ value can be ratiometrically calculated, followed by a period of continuous recording at a single wavelength. The $[Ca^{2+}]$ time course can then be calculated from the initial $[Ca^{2+}]$ value and the continuous single-wavelength trace, using a slightly adapted single-wavelength formula of Grynckiewicz et al. (1985).

A fundamental assumption in the derivation of the formula for calculating $[Ca^{2+}]$ from the initial ratiometric and subsequent single-wavelength measurements is that the total effective concentration of the fluorescent dye is expected to be constant over the recording period. This condition, unfortunately, is rarely fulfilled in reality. In experiments of extended duration (i.e., longer than 60 s), the dye concentration will gradually decrease because of photobleaching and dye leakage across the plasma membrane. Conversely, in experiments in which cell contraction occurs, the dye will become concentrated. It is mathematically possible to take the changing dye concentration into account so as to calculate a more accurate $[Ca^{2+}]$. We describe here a method of single-wavelength measurement in which a relative estimate of dye concentration is determined from fluorescence ratio pairs acquired at regular intervals and in which $[Ca^{2+}]$ is calculated taking into account the change of dye concentration. We have applied this method extensively in previous experimental work (Boitano et al., 1994; Hansen et al., 1995; Leybaert et al., 1998) but have not yet described it in extenso.

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DESCRIPTION OF THE METHOD

To obtain high temporal resolution data, the method utilizes a continuous recording of fluorescence images (e.g., at video rate) at a single wavelength. However, to reliably calculate $[Ca^{2+}]$ from these single-wavelength measurements, $[Ca^{2+}]$ images also have to be determined ratiometrically from fluorescence images at the second wavelength taken at regular intervals. A typical protocol would consist of recording images during a short exposure (t_1, e.g., 1 s or less) to excitation wavelength 1 (λ_1, e.g., 340 nm), followed by a longer period (t_2, e.g., 10 s or greater) of recording at excitation wavelength 2 (λ_2, e.g., 380 nm). This cycle is repeated continuously for the duration of the experiment. From these recordings the following fluorescence quantities are derived: F_1 from excitation with λ_1, F_2 immediately after the switch to λ_2, and $F_2(t)$ during the entire period of exposure to λ_2 (Fig. 1).

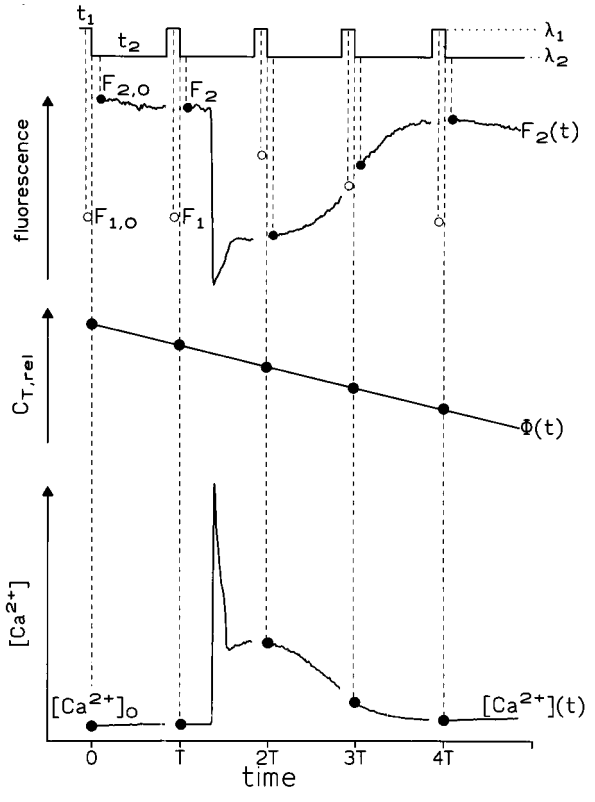

FIGURE 1 Graph illustrating the procedure for calculating $[Ca^{2+}]$ from single-wavelength measurements alternating with the sampling of ratiometric fluorescence pairs. The excitation is switched between λ_1 and λ_2, using an uneven duty-cycle protocol shown in the upper trace; t_1 and t_2 indicate the duration of each part of the cycle. The measured fluorescence quantities include the initial $F_{1,0}/F_{2,0}$ pair, subsequent F_1/F_2 pairs (○ in the fluorescence trace denote F_1; ● denote F_2), and the continuous signal $F_2(t)$ that is periodically interrupted for the measurement of F_1. The $F_{1,0}/F_{2,0}$ pair and the subsequent F_1/F_2 pairs allow calculation of the initial ($[Ca^{2+}]_0$) and subsequent $[Ca^{2+}]$ reference values, respectively. The F_2 and $[Ca^{2+}]$ reference values are used to calculate $C_{T,rel}$ values using Eq. 2. The discrete $C_{T,rel}$ data points can be described in a continuous way by the best fitting function $\Phi(t)$. $[Ca^{2+}](t)$ is then calculated from $[Ca^{2+}]_0$, $F_{2,0}$, $F_2(t)$, and $\Phi(t)$ using Eq. 4.

The F_1/F_2 fluorescence pairs form discrete data points that are generated for every interval T ($T = t_1 + t_2$; 11 s in this example), and $F_2(t)$ is a continuous function of time (except for the short interruptions of exposure to λ_1). The F_1/F_2 ratio is used to calculate $[Ca^{2+}]$, for every interval T, using the formula described by Grynckiewicz et al. (1985). These ratiometrically calculated $[Ca^{2+}]$ values are not influenced by changes in dye concentration and are referred to as $[Ca^{2+}]$ reference values. It is possible to calculate the continuous $[Ca^{2+}]$ time course, i.e., $[Ca^{2+}](t)$, from $F_2(t)$ and the $[Ca^{2+}]$ reference values. The equation for performing such a calculation is derived as follows. For a dye that decreases its fluorescence when $[Ca^{2+}]$ increases (e.g., fura-2 at 380 nm), the following formula applies (Tsien et al., 1982):

$$[Ca^{2+}] = K_d \cdot \frac{F_{max} - F_2}{F_2 - F_{min}}$$

where K_d is the dissociation constant, $F_{min} = C_T \cdot S_b$ and $F_{max} = C_T \cdot S_f$. C_T is the total concentration of the calcium dye, i.e., $C_b + C_f$, where the indices b and f denote the bound and free forms of the calcium dye. S_b and S_f are proportionality coefficients (Grynckiewicz et al., 1985). This formula can be rearranged to express C_T as a function of F_2 and $[Ca^{2+}]$:

$$C_T = F_2 \cdot \frac{([Ca^{2+}]/K_d) + 1}{([Ca^{2+}]/K_d) \cdot S_b + S_f} \quad (1)$$

Normalization of C_T relative to C_T at time 0 ($C_{T,0}$) gives the relative total dye concentration $C_{T,rel}$:

$$C_{T,rel} = \frac{C_T}{C_{T,0}} = \frac{F_2 \cdot A}{F_{2,0} \cdot A_0} \quad (2)$$

with

$$A = \frac{([Ca^{2+}]/K_d) + 1}{[Ca^{2+}]/K_d + S_f/S_b}$$

A_0 is calculated from an expression similar to that for A, but in which $[Ca^{2+}]$ is replaced by $[Ca^{2+}]_0$. $F_{2,0}$ is the F_2 value at time 0, and $[Ca^{2+}]_0$ is the $[Ca^{2+}]$ reference value at time 0 (the initial $[Ca^{2+}]$ reference value). S_f/S_b is a system constant, sometimes denoted as F_o/F_s or β, that expresses the ratio of the fluorescence at λ_2 excitation in calcium-free and dye saturating conditions. This parameter is determined, together with R_{min} and R_{max}, in a calcium-free/high-calcium ratiometric calibration procedure (R_{min}, R_{max}, and S_f/S_b are also used in the calculation of the $[Ca^{2+}]$ reference values). The quantity $C_{T,rel}$ can be calculated from each $[Ca^{2+}]$ reference value and the corresponding F_2 value, and is thus generated for every interval T.

If we make the assumption that the evolution of the total dye concentration as a function of time obeys a continuous function, i.e., a function not containing steplike changes (e.g., a linear or exponential decay profile), then the set of

discrete data points $C_{T,rel}$ can be reliably described by the fit function $\Phi(t)$ and, consequently,

$$\Phi(t) = \frac{C_T(t)}{C_{T,0}} = \frac{F_2(t) \cdot A(t)}{F_{2,0} \cdot A_0} \quad (3)$$

where $A(t)$ is given by an expression similar to that for A, but in which $[Ca^{2+}]$ is replaced by $[Ca^{2+}](t)$. Rearrangement of this equation gives the following expression for $[Ca^{2+}](t)$:

$$[Ca^{2+}](t) = K_d \cdot \frac{F_2(t)/F_{2,0} - S_f/S_b \cdot A_0 \cdot \Phi(t)}{A_0 \cdot \Phi(t) - F_2(t)/F_{2,0}} \quad (4)$$

This equation can be used to calculate $[Ca^{2+}](t)$ as a continuous function of time that is interrupted only every interval T for the measurement of F_1. The $[Ca^{2+}]$ reference values do not appear explicitly in this equation, but these values are used to calculate the $C_{T,rel}$ values from which $\Phi(t)$ is determined. The calibration parameters R_{min} and R_{max} do not appear in this equation, but they are used in the ratiometric calculation of the $[Ca^{2+}]$ reference values. The sequence of the different calculations is as follows: first, extract F_1, F_2, and $F_2(t)$ from the fluorescence recordings. Second, calculate the initial ($[Ca^{2+}]_0$) and subsequent $[Ca^{2+}]$ reference values from the F_1/F_2 pairs and the ratiometric Grynckiewicz formula. Third, calculate $C_{T,rel}$ using Eq. 2. Fourth, fit the $C_{T,rel}$ values to the function $\Phi(t)$, and fifth, use $F_2(t)$, $\Phi(t)$, $F_{2,0}$, and $[Ca^{2+}]_0$ to calculate $[Ca^{2+}](t)$ with Eq. 4.

The described protocol can be used for the calculation of $[Ca^{2+}]$ from point measurements in a series of fluorescence images or to calculate $[Ca^{2+}]$ images on a pixel-by-pixel basis. Although $\Phi(t)$ should be determined for every pixel in the image, this would involve a large number of calculations. Because often a substantial fraction of the image consists of an invariant background, we determine $\Phi(t)$ for a set of selected points within the image and calculate an average $\Phi(t)$. In principle, t_1 should be as short as possible but long enough to allow for stabilization of the excitation source and the recording device (camera lag). The duration of t_2 depends on the kinetics of the $[Ca^{2+}]$ responses and on how accurately the fit function predicts the time course of change of the total dye concentration. For the relatively long-lasting (several seconds) but kinetically fast event of calcium wave propagation between cells, we typically take 1 s for t_1 and 10–30 s for t_2. For $\Phi(t)$, a linear function often gives a reliable fit for fura-2 measurements in airway epithelial cells and astrocytes that are limited to a recording time of 10 min or less. For longer recording periods or for other cell types, an exponentially decaying function might be more appropriate. Alternatively, it is also possible to use linear interpolation between the different $C_{T,rel}$ points, in which case $\Phi(t)$ will change for every new cycle. The choice of the main excitation wavelength (λ_2) is by preference the wavelength that produces the largest change for a certain $[Ca^{2+}]$ change, but other factors, such as photobleaching effects of UV excitation, must also be considered. For fura-2 we take 380 nm as the main excitation wavelength and 340 nm as the alternative excitation (λ_1). The equations need to be adapted when λ_2 is a wavelength at which an increase in F_2 corresponds to an increase in $[Ca^{2+}]$.

Although the calculation of the $[Ca^{2+}]$ reference values is only an intermediate step in the calculation of $C_{T,rel}$, these values provide an excellent internal control, as they also report ratiometrically determined $[Ca^{2+}]$ reference values every interval T. It is recommended to plot out both $[Ca^{2+}](t)$ and the $[Ca^{2+}]$ reference values, so as to compare the two measurements (Fig. 2). Generally, there is a very good agreement between the two measured quantities, but deviations are possible, e.g., when a calcium transient occurs just at the time of the excitation switch from λ_1 to λ_2. In the case of systematic deviations it is possible to apply a correction factor so as to force, or fit with a least-squares algorithm, the $[Ca^{2+}](t)$ trace through the $[Ca^{2+}]$ reference values. Alternatively, the calculations can be performed in a slightly different way by applying the described sequence at the beginning of every excitation cycle, and by entering the $[Ca^{2+}]$ reference value at the start of that particular cycle instead of $[Ca^{2+}]_0$ in A_0 of Eqs. 2 and 4. This protocol would avoid discrepancies between the $[Ca^{2+}]$ reference and the corresponding $[Ca^{2+}](t)$ values, but could, on the other hand, introduce discontinuities into the $[Ca^{2+}](t)$ trace at the transition from one cycle to the next.

DISCUSSION

In the present work, we describe a method that allows calculation of $[Ca^{2+}]$ from single-wavelength measurements that are alternated with ratiometric measurements performed at regular intervals. The method is based on the assumption that the decay profile of total dye concentration is continuous and can be fitted by an appropriate function. The advantage of the method is that it allows high-speed (e.g., video rate and faster) monitoring of $[Ca^{2+}]$ changes without the need for high-frequency excitation switching.

An interesting aspect of the method is that calculation of the relative dye concentration ($C_{T,rel}$) is based only on measurements at the two excitation wavelengths and does not involve the measurement of any additional parameters. In principle, when the dye concentration is constant, calculation of the quantity $C_{T,rel}$ should give the same value whether it is determined from a low or a high $[Ca^{2+}]$ value of the reference point. In practice, this is not always true, and it is sometimes observed that the $C_{T,rel}$ values do deviate somewhat when calculated from reference points with a high $[Ca^{2+}]$ value. The reason for this might be related to errors introduced when F_2 approaches F_{min} (or F_{max} in the case where F_2 increases for an increase in $[Ca^{2+}]$), because at that point the relation between $[Ca^{2+}]$ and F_2 becomes very steep, as can be evaluated by calculating $d[Ca^{2+}]/dF_2$ from the single-wavelength formula given at the start of the derivation. Alternatively, erroneous

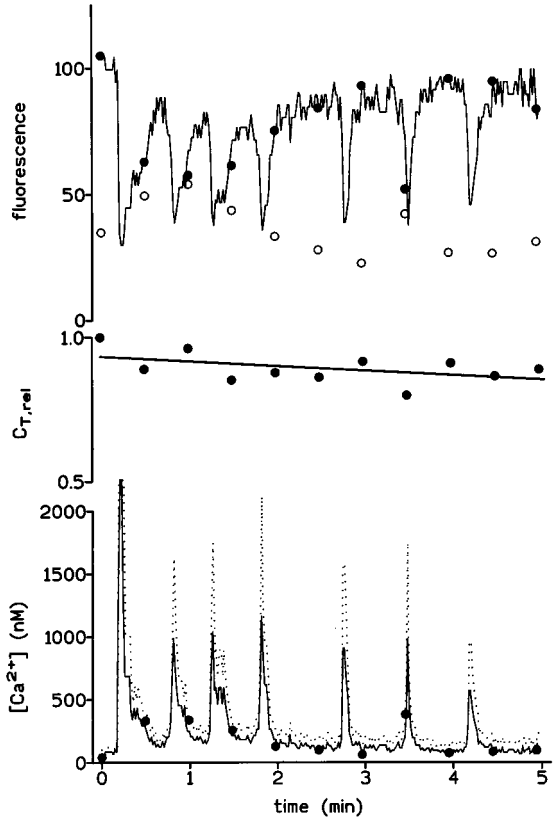

FIGURE 2 Example of an experimental recording of calcium oscillations, measured with fura-2 in cultured primary rat cortical astrocytes, and brought about by mechanical stimulation of a cell located 80 μm away. In the upper graph, fluorescence reference pairs are indicated by open and closed circles for excitation wavelengths 1 (340 nm) and 2 (380 nm), respectively. The trace represents $F_{380}(t)$ and is depicted in a continuous way without interruptions for the periodic measurements at 340 nm. The graph in the middle shows a plot of the calculated $C_{T,rel}$ data points and the best fitting linear function. The filled circles in the lower graph indicate the $[Ca^{2+}]$ reference values, and the continuous trace (*solid line*) represents the calculated $[Ca^{2+}](t)$ trace. There is good agreement between the ratiometrically determined $[Ca^{2+}]$ reference values and the calculated $[Ca^{2+}](t)$ values. The first large transient in the $[Ca^{2+}](t)$ trace is caused by the mechanically induced intercellular calcium wave passing through this particular cell, and is followed by a period of ongoing oscillations. The dotted line represents the $[Ca^{2+}](t)$ trace calculated with $\Phi(t) = 1$, i.e., without any correction for dye concentration changes or bleaching. The largest deviations between the dotted and the solid line are observed in the Ca^{2+} peaks. This is because the relation between [Ca2+] and F_{380} is very steep for high $[Ca^{2+}]$ values, so that small underestimates of F_{380} (by not taking into account dye loss or photobleaching) have profound effects on the calculated $[Ca^{2+}]$ values.

$C_{T,rel}$ values may be produced by nonlinearities in the camera and recording devices. As a result, it is better to estimate $C_{T,rel}$ from low $[Ca^{2+}]$ reference points. A valid alternative would consist of replacing the calculated relative dye concentration by the measurement of the fluorescence at the isosbestic wavelength (F_{iso} at λ_{iso}), because F_{iso} represents an equally good and perhaps more direct estimate of the relative dye concentration. In this case, λ_1 in the excitation protocol should be replaced by λ_{iso}; the $[Ca^{2+}]$ reference values can still be calculated from the F_{iso}/F_2 fluorescence ratio pairs, albeit with less precision, because the dynamic range of ratiometric measurements at these two wavelengths is lower.

An important point to be kept in mind when applying the method is the assumption of a continuous time course of change of the dye concentration, devoid of any sudden and, hence, unpredictable changes. The consequence is that any deviation of the actual dye concentration from the postulated fit function will result in the calculation of erroneous $[Ca^{2+}]$ values. Such deviations are only to be expected when the kinetics of changes in dye concentration are faster than the rate at which the reference points are sampled. A fast concentration jump can be expected, for example, in the case of mechanical cell stimulation with a micropipette, whereby stimulation is associated with some degree of plasma membrane rupture and leakage of some dye out of the cell. In principle, dye concentration changes caused by cell volume alterations can be compensated for as long as they are slow enough compared to the reference point sampling rate. In the case where cell volume changes are to be expected, linear interpolation between $C_{T,rel}$ points is preferable above searching the appropriate fit function. It is recommended that a plot be made of the $C_{T,rel}$ data points and the fit function $\Phi(t)$, so as to evaluate the quality of the assumed time course in between subsequent data points and hence to evaluate the validity of the assumption. A minor disadvantage of the method is that it does not allow expression of the result as an intermediate dimensionless quantity such as the ratio. The reason for this is that the equilibrium equation for the binding of calcium to the dye is implicitly used in the derivation of the formulas; the quantity $C_{T,rel}$ cannot be derived without this equilibrium equation. A "pseudo"-ratio, however, can be calculated by substituting $[Ca^{2+}](t)$ and $F_2(t)$ in a rearranged ratiometric Grynckiewicz formula and to calculating $F_1(t)$, followed by division of $F_1(t)$ by $F_2(t)$.

Finally, although the method is described here for the measurement of calcium with fura-2, it can be applied equally well for measurements with other dual excitation dyes such as BCECF or SBFI to determine pH or sodium, respectively. However, cellular changes of these ions are generally slower compared to calcium and may not require high temporal resolution.

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A C-program running under Windows was developed to perform the point and image calculations proposed in this manuscript. This program is available upon request from LL at the following e-mail address: luc.leybaert@rug.ac.be.

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