Quantitative Estimation of Calcium Dynamics From Ratiometric Measurements: A Direct, Nonratioing Method

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Joucla S, Pippow A, Kloppenburg P, Pouzat C. Quantitative estimation of calcium dynamics from ratiometric measurements: a direct, nonratioing method. J Neurophysiol 103: 1130–1144, 2010. First published December 2, 2009; doi:10.1152/jn.00414.2009. Measuring variations of intracellular free calcium concentration through the changes in fluorescence of a calcium-sensitive dye is a ubiquitous technique in neuroscience. Despite its popularity, confidence intervals (CIs) on the estimated parameters of calcium dynamics models are seldom given. To address this issue, we have developed a two-stage model for ratiometric measurements obtained with a charge-coupled device (CCD) camera. Its first element embeds a parametric calcium dynamics model into a fluorescence intensity model and its second element probabilistically describes the fluorescence measurements by a CCD camera. Using Monte Carlo simulations, we first show that the classical ratiometric transformation gives reliable CIs for time constants only and not baseline calcium concentration nor influx. We then introduce a direct method, which consists of fitting directly and simultaneously the fluorescence transients at both wavelengths, without any data ratioing. This approach uses a probabilistic description of the camera, leading to the construction of meaningful CIs for the calcium parameters. Moreover, using approaches inspired by constrained linear regression, we can take into account the finite precision on calibrated parameters (such as the dye dissociation constant in the cell). These key features are illustrated on simulated data using Monte Carlo simulations. Moreover, we illustrate the strength of the direct method on experimental recordings from insect olfactory interneurons. In particular, we show how to handle a time-dependent buffer concentration, thereby considerably improving our goodness of fit. The direct method was implemented in the open-source software R and is freely distributed in the CalciOMatic package.

INTRODUCTION

Calcium is known to play a crucial role in many forms of cellular activity, such as exo- and endocytosis, neurotransmitter release, cell proliferation, and cell death (for a review, see Berridge 1997). Concerning neuronal function, calcium plays a major role in induction and maintenance of short- and long-term synaptic plasticity (for reviews, see Cavazzini et al. 2005; Zucker 1999). Its ubiquitous nature has led neuroscientists to develop various techniques to explore the complex spatiotemporal organization of calcium signaling. Among them are fluorescence imaging techniques, dating back to the 1970s (Llina ́s et al. 1972; Tsien 1980), which are based on the changes of fluorescence properties of an exogenous buffer (indicator) on binding to calcium. Some dyes such as Fura-2 present different absorption spectra between their calcium-free and calcium-bound forms. This leads to ratiometric measurements, giving an estimate of the intracellular free calcium concentration with minimal processing (Grynkiewicz et al. 1985). One strength of this ratiometric transformation is that absolute estimates of calcium concentration are obtained given proper indicator calibration (Helmchen 2005). Thus the ratiometric method leads, in principle, to good estimations of various calcium dynamics parameters, such as the resting calcium level, calcium influx, or the time constant(s) of the return to baseline.

Commonly reported results using the ratiometric method consist only in optimal parameter values (Fierro et al. 1998; Helmchen et al. 1996; Kaiser et al. 2001; Muller et al. 2007). However, comparison between measurements of the same parameter under different conditions requires the estimation of confidence intervals (CIs), conveying the precision of the measurements. A difficulty and subtlety of the construction of CIs results from the fact that to obtain good fits, models must accommodate experiment-specific effects. Background fluorescence and charge-coupled device (CCD) camera properties are examples of such effects, although so-called nuisance parameters associated with these effects must also be estimated. Proper CIs of the physiological calcium parameters must therefore take into account the limited precision with which these “nuisance” parameters are known.

Here we first attempted to build CIs on calcium dynamics parameters obtained with the ratiometric method, by extending the error propagation method of Helm et al. (1997). However, this attempt turned out to be unsatisfactory. We were therefore led to develop a new “direct” method. This method embeds a calcium dynamics model within the full data generation model. The raw fluorescence data read out of the CCD camera at the two wavelengths are predicted by the model, without any data ratioing. The use of a probabilistic description of the camera led us to the construction of meaningful CIs on the calcium parameters. These intervals take into account the finite precision with which all the calibrated parameters of the model are known. These parameters are associated with both the CCD camera and the indicator. Since the direct method is nonratioing, it is sensitive to time-dependent variations of the absolute indicator concentration. A method estimating such variations was therefore developed, extending the isocoefficient method (Zhou and Neher 1993). An additional outcome of the direct method is that fitted models are amenable to rigorous goodness-of-fit tests.
The article is organized as follows: general aspects of calcium measurements with fluorescent dyes are presented first; the probabilistic CCD camera model and its experimental validation are presented next; the ratiometric and direct methods are then described and contrasted with Monte Carlo simulations; finally, experimental recordings from insect olfactory interneurons are analyzed in detail with the direct method.

Before delving into the technical description of the direct method, we conclude this introduction with a toy example showing how an accurate statistical model can lead to protocol improvements. We consider a monoexponential model of fluorescence transient, given by

$$F(t, \tau) = F_0 + \Delta F \exp(-t/\tau)$$

for which the values of $F_0$ and $\Delta F$ are known. Our goal is to estimate $\tau$ based on measurements $\{y_i\}_{i=1,...,N}$ which are modeled as realizations of Poisson distributions with parameters $\{F(t_i, \tau)\}_{i=1,...,N}$. For $F(t_i, \tau) \geq 25$, the latter can be well approximated by Gaussian distributions with variance equal to the mean, as illustrated in Fig. 1A. We then build two estimators of $\tau$, which are derived from assumed noise properties. The first estimator assumes that the noise variance is time independent (wrong noise model) and corresponds to the way calcium or fluorescence transients are fitted in the literature. On the contrary, the second estimator takes into account a noise variance equal to the signal mean (correct noise model), which corresponds to our new direct method. As detailed in Appendix A, one can theoretically show that both estimators are unbiased and derive analytical expressions of the SE of either estimator. The latter are plotted in Fig. 1B as a function of the number of measurements (uniformly distributed between $t = 0$ and $t = 5$ s). The SE of the correct estimator is lower than that of the wrong estimator, both decreasing with the sample size. Moreover, with the set of parameters chosen here ($F_0 = 100$, $\Delta F = 900$, and $\tau_0 = 1$ s), we found that a given precision on the estimation of $\tau$ is achieved with the wrong estimator by increasing the sample size by 25% compared with the correct estimator.

This result is of major importance regarding experimental protocol design, since it means that the number of illuminations could be reduced by 20% without affecting the precision of the estimations, leading to minimized cell damage. This kind of practical consideration, together with our initial goal of accurate parameter estimation, constitutes the basis of this methodological study.

Methods

Data generation

We developed a two-stage data-generation model to deal with ratiometric measurements obtained with a CCD camera. Its first element links fluorescence signals to time-dependent variations of the intracellular free calcium concentration, whereas its second element describes fluorescence measurements with a CCD camera.

Stage 1: Expression of the fluorescence intensity. The two chemical species contributing to the interesting part of the signal are the calcium-free and calcium-bound forms of the dye. Starting from Eqs. 1a and 1b of Grynkiewicz et al. (1985), we can write the fluorescence intensity at the excitation wavelength $\lambda$ as

$$F_\lambda = [(s_{\text{free},A}B) + s_{\text{bound},A}[BCa]) \phi + s_{B,A}]T_{\lambda}P$$

where $T_{\lambda}$ is the exposure time (in s), $P$ is the number of pixels of the region of interest (ROI), $[B]$ and $[BCa]$ are the concentrations of the free and calcium-bound buffer (in $\mu M$), $s_{\text{free},A}$ and $s_{\text{bound},A}$ are two coefficients “transforming” concentrations of free and bound buffer into fluorescence intensities [in count/$(\mu M \cdot \text{pixel} \cdot s)$], $s_{B,A}$ is the experiment-specific background fluorescence [in count/($\text{pixel} \cdot s$)], and $\phi$ is a dimensionless experiment-specific parameter.

Parameter $\phi$ is used to lump together factors expected to change from experiment to experiment, but to remain constant within a single experiment (for instance, the excitation lamp intensity, the thickness of the neuronal process actually excited by the UV light, or the depth of the neurite, implying a change in the optical paths of the excitation and fluorescent lights). The experiment-specific background parameter $s_{B,A}$ takes into account the fact that the ratio of excited extracellular to intracellular volume might change from experiment to experiment. It also includes the autofluorescence of the intracellular organelles present on the optical path, as well as the dark current contribution (see Stage 2: A CCD Camera Model).

Using ratiometric measurements, we can rewrite Eq. 1 in terms of familiar parameters. From here forward we will refer to the use of Fura-2 with two excitation wavelengths centered on 340 and 380 nm, respectively. Each measurement thus becomes a set of two measurements performed almost simultaneously. Assuming that the three key species (free calcium, free Fura, and calcium-bound Fura) are in chemical equilibrium, we can write, from the law of mass action...
where $K_d$ is the dissociation constant of Fura (in μM) and $[B_{380}]$ is the total concentration of Fura in the cell. Substituting these expressions into the fluorescence model, we get

$$F_{380} = \left\{ \frac{[B_{380}] \phi}{K_d + [Ca^{2+}]} \right\} \left( S_{380, \text{free}} K_d + S_{380, \text{bound}} [Ca^{2+}] + s_{B,380} \right) T_{e,380} P$$

$$F_{340} = \left\{ \frac{[B_{340}] \phi}{K_d + [Ca^{2+}]} \right\} \left( S_{340, \text{free}} K_d + S_{340, \text{bound}} [Ca^{2+}] + s_{B,340} \right) T_{e,340} P$$

We then introduce the following variables

$$R_{\text{min}} = \frac{S_{340, \text{free}}}{S_{340, \text{bound}}}$$

$$R_{\text{max}} = \frac{S_{380, \text{free}}}{S_{380, \text{bound}}}$$

$$R_{380} = \frac{S_{380, \text{free}}}{S_{380, \text{bound}}}$$

$$K_{\text{eff}} = R_{380} K_d$$

where $R_{\text{min}}$ and $R_{\text{max}}$ are the minimum and maximum measurable fluorescence ratios and $K_{\text{eff}}$ is the effective dissociation constant of Fura within the cell. All these parameters are obtained from calibration experiments. After introducing the expression of these calibrated parameters into the fluorescence model, we get

$$F_{340} = \left\{ \frac{[B_{340}] \phi}{K_d + [Ca^{2+}]} \right\} (R_{\text{min}} K_{\text{eff}} + R_{\text{max}} [Ca^{2+}] + s_{B,340}) T_{e,340} P$$

$$F_{380} = \left\{ \frac{[B_{380}] \phi}{K_d + [Ca^{2+}]} \right\} (K_{\text{eff}} + [Ca^{2+}] + s_{B,380}) T_{e,380} P$$

where factor $S_{380, \text{bound}}$ has been absorbed in $\phi$. In the absence of fluorescent dye, the Eq. 2 system reduces to background fluoresences

$$F_{B,340} = s_{B,340} T_{e,340} P_B$$

$$F_{B,380} = s_{B,380} T_{e,380} P_B$$

where $P_B$ is the number of pixels used for the background region (potentially different from $P$ if the background region does not coincide with the ROI).

STAGE 2: A CCD CAMERA MODEL. Once known, the ideal expressions of the fluorescence signals as a function of the intracellular free calcium concentration, we can sketch the operation of a CCD camera as described in Mullikin et al. (1994), van Vliet et al. (1998), and Janesick (2001). Pixels of a CCD camera “move” one electron into a potential well on absorption of one photon. This process is characterized by Poisson statistics of parameter $F$. As described by Eq. 1, in the case of the CCD pixel, $F$ is proportional to the intensity of the light falling on the pixel and to the exposure time. Electrons can also spontaneously appear in the potential well due to thermal agitation, giving rise to the dark current. The distribution of these electrons is also Poisson with a parameter proportional to the exposure time. For $P$ pixels, by property of the Poisson distribution, the Poisson parameter is equal to $P$ times the Poisson parameter for 1 pixel, which explains the form of Eq. 1. Moreover, the capacity of the potential well is finite. After exposure, each pixel (or group of binned pixels) is read, resulting in an analog (i.e., continuous) signal that involves the measurement of a voltage $V$ at the terminals of a capacitor. This electron-to-voltage conversion is noisy, “adding” an independent Gaussian readout noise to the signal (van Vliet et al. 1998). Finally, the voltage reading of each pixel is multiplied by a gain factor $G$ (typically $<1$) before being digitized to give the output in analog-to-digital units ($adu$). We thus get a three-phase data-acquisition process

$$N_{\text{photons}} \sim \text{Poisson}(F)$$

$$V \sim \text{Norm}(N_{\text{photons}}, \sigma^2_{\text{read}})$$

$$adu = \lceil GV \rceil$$

where $N_{\text{photons}}$ is the number of photons absorbed by the pixel(s) of the camera, “$\sim$” means that the left-hand-side random variable has the distribution given on the right-hand side, $F$ has the expression given in the previous section, Poisson ($F$) stands for a Poisson distribution with parameter $F$, $\sigma^2_{\text{read}}$ is the variance of the readout process, Norm ($\mu$, $\sigma^2$) stands for a normal distribution with mean $\mu$ and variance $\sigma^2$, and $\lceil \cdot \rceil$ stands for the integer part of $x$ (the largest integer $\leq x$).

For a sufficiently high parameter ($\geq 25$), the Poisson distribution can be well approximated by a Gaussian distribution with variance equal to the mean. Consequently, for high photon counts, the measured $adu$ signal can be modeled as following a Gaussian distribution

$$adu \sim \text{Norm}[GF, G^2(F + \sigma^2_{\text{read}})]$$

Experimental characterization of a CCD camera. We performed experiments to characterize the gain and readout noise of an Imago/SensiCam CCD camera (Till Photonics, Gräfelfing, Germany). Fluorescence measurements were made using a fluorescent plastic slide (Chroma-Gesellschaft, Stuttgart, Germany). Ten exposure times were used, from 10 to 100 ms. For each duration, 100 consecutive measurements were performed with a cycle time of 200 ms. Means and variances were calculated on the 100 measurements for each pixel and then averaged over the 60 × 80 pixels of the camera. The 10 variances were then linearly fitted against the corresponding 10 means. Following Eq. 5, the gain of the CCD camera $(G)$ corresponded to the slope of the linear fit, whereas the variance of the readout noise $(\sigma^2_{\text{read}})$ was given by the intercept divided by the square of the slope (see Fig. 2A). We found a gain of 0.146 and a readout noise of SD $\sigma_{\text{read}}$ = 16.4 photoelectrons.

Inference of parameters

In this study, our goal was to build meaningful CIs on estimated calcium dynamics parameters. For this purpose, we first recall the “classical” method, based on the ratiometric transformation of the experimental data, and address an important limitation of this method regarding the construction of CIs. Then, we propose a new direct method, simply working on the original fluorescence signals.

Our experimental protocols include: first, one (or more) set of two transients obtained once Fura has been loaded into the cell. In the absence of calcium, $F$ takes the form $380$, $340$, and $340$, as $0$ performed at 340 and 380 nm ({$adu_{B,340}, adu_{B,380}, j = 1, \ldots, 3$}; second, one (or more) set of two transients obtained once Fura has been loaded into the cell ({$adu_{B,340}, adu_{B,380}, j = 1, \ldots, 3$}). Formally, these measurements are samples of Poisson random variables with parameters $F_{340}$ and $F_{380}$, given by Eq. 2 (respectively with parameters $F_{B,340}$ and $F_{B,380}$ given by Eq. 3 for the background measurements).

METHOD 1: THE RATIOMETRIC METHOD. The experimental ratios are defined as

$$\frac{F_{380}}{F_{340}}$$

$\frac{340}{380}$
experimental calcium time course \( \text{ca} \), one can try to model it with a parametric function of time \( [\text{Ca}^{2+}](t, \theta_{\text{calib}}) \), for instance, with a monoexponential model. The parameters \( \theta_{\text{calib}} \) of this model are estimated by minimizing the following residual sum of squares (RSS)

\[
\text{RSS}_{\text{calib}}(\theta_{\text{calib}}) = \{ca - [\text{Ca}^{2+}](t, \theta_{\text{calib}})\}^T \cdot \Omega^{-1} \cdot \{ca - [\text{Ca}^{2+}](t, \theta_{\text{calib}})\}
\]

where \( \Omega \) stands for the approximate covariance matrix of the calcium signal \( \text{ca} \) deduced from Eq. 8. As detailed in Appendix B, a theoretical expression of the covariance matrix can be deduced from a first-order Taylor expansion of Eq. 8 and an approximate \( \Omega \) can then be calculated from the experimental measurements of \( \text{adu}_{\text{B,340}} \) and \( \text{adu}_{\text{B,380}} \). Equation 9 assumes that the distribution of \( \text{ca} - [\text{Ca}^{2+}](t, \theta_{\text{calib}}) \) is a multivariate Gaussian. This assumption must be checked and must be approximately correct to obtain reliable CIs. We show in Appendix B that such an approximation is not correct for our typical recording settings. Consequently, the CIs estimated using the ratiometric method are not meaningful, as illustrated in Fig. 3. In particular, the CIs are quite underestimated when the actual values of the calibrated parameters are not equal to their experimental mean value (Fig. 3B).

**METHOD 2: A DIRECT METHOD.** This limitation of the ratiometric method can be overcome using a new direct method—simply working on the original fluorescence measurements (\( \text{adu} \)) that does not require any data ratioing. It consists only in embedding a calcium

![FIG. 2. Validation of the charge-coupled device (CCD) camera model and the square-root transformation. Fluorescence measurements were made using a fluorescent plastic slide. Ten exposure times were considered, from 10 to 100 ms. For each duration, 100 consecutive measurements were performed with a cycle time of 200 ms. Means and variances were calculated on the 100 measurements for each pixel of the camera and then averaged over all pixels of the CCD camera. A: as expected from Eq. 5, the variance of the adu increases linearly with its mean. Fitting a linear relationship between \( \sigma^2(\text{adu}) \) and \( \text{adu} \) (dashed gray line) gives the gain of the camera (G, equal to the slope) and the variance of the readout noise (\( \sigma^2_{\text{ro}} \), equal to the intercept multiplied by the square of the slope). B: taking the square root of the raw fluorescence measurements helps to stabilize the variance for high photon counts (\( \text{adu} \geq 1000 \)). The dashed gray line represents the theoretical values of the variance of the square-rooted \( \text{adu} \) (equal to \( 10 \times \text{adu}^2 \)), and the variance stabilizes to \( G/4 \) (horizontal dashed gray line).

\[
\begin{align*}
\sigma^2_{\text{ro}}(\text{adu}) & = \frac{\text{adu}^2}{G^2/4} \\
\text{adu} & = \frac{\text{adu}^{0.5}}{G^{0.5}}, \quad \text{adu}^{0.5} \text{ is the square-root transformation of the } \text{adu}
\end{align*}
\]

where \( G^{-1} \text{adu}_{\text{B,340}} \) and \( G^{-1} \text{adu}_{\text{B,380}} \) stands for the mean background measurement at \( \lambda \). From Eqs. 2 and 3, we can rewrite Eq. 6 as

\[
\text{adu}^{0.5} = \frac{R_{\text{max}} - R_{\text{min}}}{K_{\text{eff}} + \text{ca}} \]

which allows us to retrieve the estimated intracellular free calcium concentration

\[
\text{ca} = K_{\text{eff}} \text{adu}^{0.5} - R_{\text{min}}
\]

In this expression, the calibrated parameters are set to their experimental mean values (respectively, \( R_{\text{min}}, R_{\text{max}}, \) and \( K_{\text{eff}} \)). Knowing the

![FIG. 3. Validation of the direct method using Monte Carlo simulations. In all, 1,000 fluorescence transients were drawn from Poisson distributions, according to the parameters given in Table 2. A: simulations were run with all calibrated parameters set to their mean value. The model parameters were estimated either by fitting the calcium transients arising from the ratiometric transformation (\( \Delta \)) or the square-rooted fluorescence transients ("original" direct method, •). The number of times that the 95% confidence interval provided by the fit contained the true value of the parameter is reported for each method and each parameter and can be compared with the 0.025 and 0.975 quantiles of the binomial distribution (dashed gray lines). B: simulations were run with calibrated parameters independently drawn from Gaussian distributions at each trial. Fits were first performed with the ratiometric method, with all calibrated parameters set to their mean value, which leads to wrong estimations of the "amplitude" parameters \( \text{ca} \) and \( \text{dc} \) (•). Then, the direct method was "extended" by estimating simultaneously the actual values of \( \theta_{\text{calib}} \), which gave reliable 95% confidence intervals.
dynamics model into the fluorescence model (Eqs. 2 and 3) and optimizing the parameters to fit the experimental fluorescence transients. Since our aim was to build meaningful CIs, we have to look carefully at the statistical properties of these fluorescence signals. In following Eq. 5, the variance of the adu values depends linearly on their expected mean value. One could use this property to calculate approximate variances of the adu measurements and fit the model parameters by minimizing a weighted least-squares criterion, as is done in the ratemetric method. Here, we prefer working with transformed signals for which the variance has been stabilized, i.e., made independent of the amplitude of the fluorescence signals.

The square root transformation. In the special case of a camera displaying a gain of 1 and a readout noise with a variance of 0, the variance of the adu values is equal to their mean (Poisson distribution). In 1936, Bartlett proposed to use the square-root transformation, i.e., to work on the square root of the data (√adu). This square-root transformation stabilizes the variance of a Poisson distribution to 1/4 as soon as the Poisson parameter is large enough (in practice, the variance of the square-root Poisson distribution differs from 1/4 by <2% as soon as its parameter exceeds 20). With this transformation, the direct least-squares criterion is minimized to

$$\text{RSS}_{\text{direct}}(\theta_{\text{exp}}, \theta_{\text{exp}}) = \sum_{i=1}^{i} \left( \frac{\sqrt{\text{adu}_{B,340,i}^{\text{direct}}}}{\sqrt{\text{F}_{340}(\theta_{\text{exp}})}} - \frac{\sqrt{\text{F}_{340}(\theta_{\text{exp}})}}{\sqrt{\text{adu}_{B,340,i}^{\text{direct}}}} \right)^2$$

$$+ \sum_{i=1}^{i} \left( \frac{\sqrt{\text{adu}_{B,380,i}^{\text{direct}}}}{\sqrt{\text{F}_{380}(\theta_{\text{exp}})}} - \frac{\sqrt{\text{F}_{380}(\theta_{\text{exp}})}}{\sqrt{\text{adu}_{B,380,i}^{\text{direct}}}} \right)^2$$

$$+ \sum_{i=1}^{i} \left( \frac{\sqrt{\text{adu}_{340,i}^{\text{direct}}}}{\sqrt{\text{F}_{340}(\theta_{\text{exp}})}} - \frac{\sqrt{\text{F}_{340}(\theta_{\text{exp}})}}{\sqrt{\text{adu}_{340,i}^{\text{direct}}}} \right)^2$$

$$+ \sum_{i=1}^{i} \left( \frac{\sqrt{\text{adu}_{380,i}^{\text{direct}}}}{\sqrt{\text{F}_{380}(\theta_{\text{exp}})}} - \frac{\sqrt{\text{F}_{380}(\theta_{\text{exp}})}}{\sqrt{\text{adu}_{380,i}^{\text{direct}}}} \right)^2$$

(10)

where \(\theta_{\text{exp}}\) are the experiment-specific parameters to estimate; \(\{\phi, \gamma_2, \gamma_3, \gamma_4, \gamma_5, \gamma_6\}\). In this expression, \(F_{340}(\theta_{\text{exp}})\) and \(F_{380}(\theta_{\text{exp}})\) are given by Eq. 3, whereas \(F_{340}(\theta_{\text{exp}}, \theta_{\text{exp}})\) and \(F_{380}(\theta_{\text{exp}}, \theta_{\text{exp}})\) are given by Eq. 2, where \([\text{Ca}^{2+}]\) has been replaced with the model: \([\text{Ca}^{2+}] + (\theta_{\text{calc}})\).

In practical situations (see the experimental characterization of our CCD camera, stage 2), the gain of the camera is strictly <1 and the readout noise has a nonzero variance. From Eq. 5, we can deduce the law of the square-rooted adu

$$\sqrt{\text{adu}} \sim \text{Norm} \left( \sqrt{\text{GF}}, \frac{G}{\sqrt{4}} \right)$$

(11)

With this square-root transformation, the variance stabilizes only for high adu values (≥1,000 for the data obtained during the camera calibration experiments; see Fig. 2B). To face the stabilization problem encountered at “low” adu, we now use a modified square-root transformation, taking into account the characteristics of the camera

$$\sqrt{\text{adu}} + \sqrt{\text{G} \sigma_{\text{str}}} \sim \text{Norm} \left( \sqrt{\text{GF} + \sigma_{\text{str}}^2}, \frac{G}{\sqrt{4}} \right)$$

(12)

This transformation, which leads to a variance stabilization irrespective of the fluorescence level (see Fig. 2C), will be referred to throughout as the “square-root transformation.” We emphasize that once the square-root transformation has been performed, one is brought back to a standard nonlinear regression setting, i.e., the noise of the signals to fit is independent and identically distributed (iid) over time. This allows an accurate construction of confidence intervals (Bates and Watts 1988; Ruppert et al. 2003), which will be further illustrated in RESULTS (see Fig. 3).

Taking into account the uncertainty of calibration measurements. In the “original” direct method, all calibrated parameters \(\theta_{\text{calc}} = [R_{\text{max}}, R_{\text{min}}, K_{\text{calc}}, k_c]\) are set to their experimental mean value, which were obtained by repeating calibration measurements many times. To take into account the uncertainty of these parameters, we propose an approach inspired by constrained linear regression. In this “extended” method, each parameter \(\theta_{\text{calc}}\) is allowed to vary around its experimental mean value \(\theta_{\text{calc},i}\), so that the fluorescence signals \(F_{340}\) and \(F_{380}\) now depend on \(\theta_{\text{calc},i}\). The approximate variances of the calibrated parameters are constrained by their experimental SE \(\sigma(\theta_{\text{calc},i})\), which leads us to minimize the following weighted least-squares criterion

$$\text{RSS}_{\text{direct+calc}}(\theta_{\text{calc},i}, \theta_{\text{exp}}, \theta_{\text{calc},i}) = \frac{4}{G} \text{RSS}_{\text{direct}}(\theta_{\text{calc},i}, \theta_{\text{exp}}, \theta_{\text{calc},i})$$

$$+ \sum_{i=1}^{i} \frac{(\theta_{\text{calc},i} - \hat{\theta}_{\text{calc},i})^2}{\sigma(\theta_{\text{calc},i})^2}$$

(13)

where the weight \(4/G\) arises from the fact that the variance of the square-rooted fluorescence signals is \(G/4\).

Accounting for time-dependent variations of \([\text{B}T]_j\). The “extended” direct method now allows the estimation of all parameters of the fluorescence model, including the Fura concentration, through the \([\text{B}T]_j\) factor. During one experiment, i.e., during the acquisition of a single calcium transient, \(\phi\) should remain constant, except when major preparation movements occur. On the contrary, \([\text{B}T]_j\) can change, especially when the experiments are performed with patch-clamp recordings and when the calcium transients are evoked shortly after break-in. To properly fit such transients and get accurate estimations of calcium dynamics, we need to handle these time-dependent variations of \([\text{B}T]_j\). The simplest way to do this is to simultaneously record a fluorescence transient at 360 nm (the Fura-2 isosbestic wavelength). Indeed, this signal is proportional to \([\text{B}T]_j\) and could advantageously replace the latter in the expressions of \(F_{340}\) and \(F_{380}\).

In a situation where recordings at 360 nm were not performed during the transients, but at other instances (e.g., during a loading curve), we propose to handle potential time-dependent variations of \([\text{B}T]_j\) through an approach derived from the isocoefficient method (Zhou and Neher 1993). In that study, the authors argued that there exists a linear relationship between the fluorescence signals obtained at 340 and 380 nm. More precisely, there exist two coefficients \(\alpha\) and \(\beta\) such that

$$\frac{F_{340} - \text{adu}_{340}}{T_{340}} = \frac{F_{380} - \text{adu}_{380}}{T_{380}} + \beta$$

$$= \alpha$$

$$\beta = [\text{B}T]_j (\theta_{\text{calc}} R_{\text{max}} - R_{\text{min}})$$

(14)

Including Eqs. 2 and 3 of the fluorescence model into this expression leads to theoretical values of \(\alpha\) (the isocoefficient) and \(\beta\)

$$\alpha = \frac{R_{\text{max}} - R_{\text{min}}}{R_{380} - 1}$$

(15a)

$$\beta = \frac{[\text{B}T]_j \phi R_{380} (R_{\text{max}} - R_{\text{min}})}{R_{380} - 1}$$

(15b)

The key features of these equations are that the value of \(\alpha\) depends neither on \([\text{Ca}^{2+}]\) nor on \([\text{B}T]_j\) and that the value of \(\beta\) does not depend on \([\text{Ca}^{2+}]\). However, it is directly proportional to \([\text{B}T]_j\), which means that if we are able to estimate \(\alpha\), we will be able to follow the variations of \([\text{B}T]_j\) with time.

In APPENDIX C, we propose a three-wavelength protocol, based on fluorescence measurements performed at 340, 380, and 360 nm, to get
an estimate of both $\bar{a}$ and $\sigma^2(\bar{a})$. We then include this estimation of $\alpha$ in the fluorescence model to fit the transient of interest. More precisely, in Eq. 2, we replace the single parameter $[B_f]$ with $\exp + \alpha \cdot \text{adu}_{\text{calib}}$, and the final weighted least-squares criterion to minimize becomes

$$\text{RSS}_{\text{direct,calib}}(\theta_{\text{calib}}, \theta_{\exp}, \theta_{\text{adu}}, \alpha) = \text{RSS}_{\text{direct,calib}}(\theta_{\text{calib}}, \theta_{\exp}, \theta_{\text{adu}}, \alpha) + \frac{(a - \bar{a})^2}{\sigma^2(\bar{a})} \quad (16)$$

This approach will be referred to as the “full” direct method. We note that the expressions of $F_{340}$ and $F_{380}$ should not depend explicitly on $\text{adu}_{\text{calib}}$ and $\text{adu}_{\text{calib}}$, since the latter are the transients to be fitted. Thus we considered smoothed versions of these signals (obtained using constrained B-splines; Ng and Maechler 2009), consistent with the fact that $[B_f]$ is expected to vary smoothly during the transient (at least after the depolarizing voltage step). An illustration of the smoothed $\text{adu}_{\text{calib}}$ and $\text{adu}_{\text{calib}}$ is given in Fig. 5.

**Fitting a parametric model of calcium dynamics**

We focused on a parametric model of calcium dynamics where $[\text{Ca}^{2+}]$ decays monoexponentially following a stimulation occurring at $t_{\text{ON}}$. This time course is modeled as

$$[\text{Ca}^{2+}](t, \theta_{\text{calib}}) = \begin{cases} C_{\text{calib}} & \text{if } t < t_{\text{ON}} \\ C_{\text{calib}} + \Delta \text{Ca} \exp - \frac{t - t_{\text{ON}}}{\tau} & \text{if } t \geq t_{\text{ON}} \end{cases} \quad (17)$$

with $\theta_{\text{calib}} = [C_{\text{calib}}, \Delta \text{Ca}, \tau]$. As introduced earlier, the model parameters were estimated by minimizing a least-squares criterion dependent on the chosen method (ratiometric or direct). This was done in R (R Development Core Team 2009). In the ratiometric case, we used the optim function with the “BFGS” (Broyden–Fletcher–Goldfarb–Shanno) algorithm (Nocedal and Wright 1999). In the direct case, we used the nlsm function with its default Gauss–Newton algorithm (Bates and Maechler 2009). Consistent with the fact that $[B_f]$ is expected to vary smoothly during the transient (at least after the depolarizing voltage step). An illustration of the smoothed $\text{adu}_{\text{calib}}$ and $\text{adu}_{\text{calib}}$ is given in Fig. 5.

**Experimental protocol.** Experiments were performed in the first central relay, the antennal lobe, of the cockroach *Periplaneta americana* olfactory system. Details of the experimental procedure can be found in Pippow et al. (2009). Briefly, whole cell recorded neurons were loaded with Fura-2 via the patch-clamp pipette ($[B_f]_{\text{pipette}} = 200 \mu M$). Loading curves were determined by illuminating the preparation at 360 nm every 30 s for about 20 min (exposure time: 7 ms). Three calcium transients were elicited during the loading curve by stepping the voltage-clamped membrane potential from −60 to −5 mV for 50 ms. During the transients, fluorescence images were acquired at 340 and 380 nm every 75 ms for 12 s (exposure times: 15 and 6 ms). Background fluorescence images of the whole preparation were acquired at each of the three wavelengths before the whole cell configuration was established (break-in). ROIs consisted of rectangular zones centered on the cell bodies, in which $[\text{Ca}^{2+}]$ was uniform, as assessed by plots similar to Fig. 5 of Neher and Augustine (1992).

**Fluorescence Transients Analysis.** A monoexponential calcium dynamics model was fitted with the direct method described earlier, with or without taking into account the uncertainty of calibration measurements and time-dependent variations of the intracellular Fura concentration (Figs. 4 and 6). For the latter, we estimated $\alpha$ based on the three-wavelength protocol described in
Calcium transients were induced in olfactory interneurons of the cockroach *Periplaneta americana* by somatic depolarization at \( t_{\text{ON}} \). The fluorescence transients recorded at 340 and 380 nm were fitted simultaneously using the **direct** method. A: as shown on the fit of \( \Delta \text{fluorescence} \), fitting only \( \theta_{\text{calib}} \) and \( \theta_{\text{exp}} \) neither allows a good estimation of the absolute fluorescence levels nor a good estimation of the time constant of the return to baseline. B: the fit of the latter can be improved by simultaneously fitting \( \theta_{\text{calib}} \) but a slight drift remains in the residuals (bottom). Moreover, the fluorescence baseline remains badly fitted.

**APPENDIX C**, by using the three \( \text{adu}_{340} \) and \( \text{adu}_{380} \) transients evoked during the loading curve. Using smoothing splines, we extrapolated the \( \text{adu}_{380} \) recordings of the loading curve to the latencies of these three transients. \( \alpha \) was determined using only the measurements performed before the voltage stepping (at \( t_{\text{ON}} \)) and the last measurement of each transient.

As shown in Figs. 4 and 6, the fluorescence peak was not reached instantaneously after the voltage step. This may result from the fact that the three species of interest (free calcium, free Fura, and calcium-bound Fura) were not in chemical equilibrium during the rising phase of the transient. Fits were thus performed by skipping this rising phase as well as several samples after the peak.

**TESTING THE GOODNESS OF A FIT.** To test whether a model satisfactorily fits the experimental data, we performed two basic tests based on the characteristics of the noise, which is assumed to be independently and identically distributed (iid) throughout the experiment, following a Gaussian distribution:

1) The independence was tested by computing the autocorrelation function (ACF) of the fit residuals, i.e., the cross-correlation of the residuals vector with itself, shifted by 0, one, or more time samples. To satisfy this test, 95% of the values taken by the ACF should fall between \( \pm n(1-q)/2\sqrt{n} \), where \( n \) is the quantile function of a Gaussian distribution of mean = 0 and SD = 1 (see Fig. 6C).

2) The adequation to a Gaussian distribution was assessed by a quantile–quantile (q–q) plot, which consists in plotting the residual quantiles against the theoretical quantiles of a normalized centered Gaussian distribution. To satisfy the test, the q–q points should fall approximately along a straight line whose slope is equal to the SD of the residuals (see Fig. 6D).

**CalcioMatic package**

The ratiometric and direct methods described herein have been implemented in the R environment (R Development Core Team 2009). All codes have been gathered into the CalcioMatic package, which can be freely downloaded from the Comprehensive R Archive Network (CRAN) website (http://cran.r-project.org/). The package includes easy-to-use functions to simulate data and fit simulated or experimental data with either of the two methods. Dedicated plot functions have also been implemented to visualize raw and fitted data and to test the goodness of fit. A brief presentation of how to use the different functions of the CalcioMatic package is given in the package documentation and tutorial available from the CRAN website.

**RESULTS**

**Validation of the direct method: Monte Carlo simulations**

As described in METHODS, we used a Monte Carlo approach to estimate the coverage probability of the confidence intervals (CIs) provided by the ratiometric and direct methods. Simulations were run with the parameters given in Table 1.

In a first step, we considered ideal situations where all calibrated parameters were known and equal to the mean value obtained from the calibration experiments. We generated 1,000 fluorescence transients according to the Poisson statistics, which were fitted with both the ratiometric and direct methods. For each method and each parameter, we counted the number of times \( N_{ \text{c} } \) that the true value of the parameter fell into the 95% CI provided by the fit. This quantity was then compared with the 0.025 and 0.975 quantiles of the binomial distribution with size = 1,000 and success probability \( P = 0.95 \) (936 and 963, respectively; see the gray confidence bands in Fig. 3A).

The \( N_{ \text{c} } \) values obtained with the ratiometric method (\( \gamma \)) fell within the confidence bands for the parameter \( \tau \) only and were outside this band for \( \text{Ca}_{\text{0}} \) and \( \Delta \text{Ca} \), the distance to this band being particularly large for \( \text{Ca}_{\text{0}} \). This means that the coverage probability of these “confidence intervals” was below the nominal coverage probability (95%). Conversely, the \( N_{ \text{c} } \) values obtained with the “original” direct method (\( \bullet \)) fell within the confidence band for all \( \theta_{\text{calib}} \) and \( \theta_{\text{exp}} \) parameters, illustrating the accurate construction of meaningful 95% confidence intervals with this method.

In a second step, we mimicked experimental situations where calibrated parameters are not necessarily equal to their experimental mean value. For that purpose, we performed simulations where, at each trial, calibrated parameter values were drawn from Gaussian distributions with mean and SD set, respectively, to \( \theta_{\text{calib}} \) and \( \text{se}(\theta_{\text{calib}}) \). As before, 1,000 noisy fluorescence signals were generated and fitted using both the ratiometric and the direct methods. Concerning the ratiometric method, the equivalent calcium transients deduced from Eq. 8 were obtained with \( R_{\min}, R_{\max} \), and \( K_{\text{eff}} \) set to \( R_{\min} \cdot R_{\max} \), and \( K_{\text{eff}} \), respectively. In a first approach, we modified the covariance matrix \( \Sigma \) to take into account the measurement uncertainty on \( R_{\min}, R_{\max}, \) and \( K_{\text{eff}} \) as explained in **APPENDIX B**. Doing so, the regression algorithm returned finite optimal values for \( \text{Ca}_{\text{0}}, \Delta \text{Ca}, \) and \( \tau \), but the SE on the two latter were infinite. More specifically, the estimated calcium transient was constant and we were not able to build confidence intervals for \( \Delta \text{Ca} \) and \( \tau \). For that reason, we ended up not including the
contribution of the calibrated parameters in the covariance matrix.

Using this new simulation paradigm, we found that the \( N_{s} \) value obtained for \( \tau \) with the ratiometric method was still within the confidence band (Fig. 3B, ▼). On the contrary, the calcium baseline and influx were very badly estimated, the coverage probability of their “confidence intervals” decreasing dramatically compared with former simulations (\( N_{s} = 367 \) vs. 533 for \( Ca_{0} \) and \( N_{s} = 347 \) vs. 893 for \( \Delta Ca \)). Conversely, the confidence intervals provided by the “extended” direct method were found to be relevant for all calibrated parameters set to their mean value and tried with all calibrated parameters set to their mean value and tried for \( \Delta Ca \) estimation, we were then able to follow the variations of \( Ca_{0} \) during the transient, which are proportional to \( adu \) (analog to digitals units).

We then used the “extended” direct method, by optimizing simultaneously the value of the calibrated parameters. As shown in Fig. 4B, this approach improved the fit of the decay, but a slight drift remained in the residuals. Moreover, the estimation of the fluorescence baseline was still off.

In these two first approaches, the total concentration of Fura-2 in the soma (\( [B_{r}] \)) was considered constant during the transient. However, since a depolarization was induced with a patch-clamp pipette containing Fura (which is a charge carrier), its concentration within the cell was susceptible to increase following the depolarization. To check this hypothesis, we used the three-wavelength protocol described in METHODS. We then used the “extended” direct method, by optimizing simultaneously the value of the calibrated parameters. As shown in Fig. 4B, this approach improved the fit of the decay, but a slight drift remained in the residuals. Moreover, the estimation of the fluorescence baseline was still off.

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Fitting calcium dynamics in olfactory interneurons

After validating the direct method on simulated data, we looked into the analysis of experimental fluorescence transients. Experiments were performed in the first olfactory relay of the cockroach Periplaneta americana, to characterize the endogenous binding capacity of different classes of neurons. Our goal here was to show how much information can be provided by the direct method. Thus we chose to focus our analysis on a single representative experiment.

In a first approach, we used the “original” direct method with all calibrated parameters set to their mean value and tried to fit a monoexponential model of calcium dynamics. We skipped the part of the signals corresponding to the first second after somatic depolarization. As illustrated in Fig. 4A, the fluorescence baseline, as well the fluorescence jump at \( t_{on} \) and the return to baseline were badly estimated (raw and fitted data are reported at 340 nm, but similar results were obtained at 380 nm, both data being simultaneously fitted).
performed using this “full” direct method (Fig. 6), with which we were now able to accurately fit the baseline, as can be seen in Fig. 6, A and B. The adequacy of the monoexponential calcium dynamics model was further quantified with the autocorrelation function (ACF; Fig. 6C) and a q–q plot (Fig. 6D). For both diagnostic plots, gray bands corresponded to pointwise 95% confidence intervals. The ACF indicates that there is no temporal structure in the residuals, i.e., the noise is distributed independently along time. The q–q plot shows that the noise is identically distributed, following a Gaussian distribution with mean equal to 0 and SD equal to the slope of the straight line passing through the q–q points (0.209). We note that the slope of this line, corresponding to the SD of the residuals, was very close to its expected value (0.209), close to the expected theoretical value ($\sqrt{2} = 0.191$), which confirmed the quality of the monoexponential model to predict these experimental data. We emphasize that this accurate fit did not result from an important tail-off of the distribution and is somewhat different from the parameter of this distribution, leading to shifts in both numerators and denominators of Eq. 6. These offsets then dramatically affect the estimation of $C_{0\text{B}}$, but not that of $\Delta C_{0}$ and $\tau$, which are baseline independent.

In more realistic situations where calibrated parameters are known with a finite precision, and thus not necessarily equal to their experimental mean value, $\tau$ remained the only parameter to be accurately estimated with the ratiometric method, whereas the coverage probability of the CIs on $C_{0\text{A}}$ and $\Delta C_{0}$ were dramatically underestimated (Fig. 3B, ▼). To shed light on the latter result, we consider again Eq. 8 and see how the estimation of the calcium dynamics depends on the calibrated parameters. The minimal and maximal fluorescence ratios are generally well known [in our calibration experiments, we had obtained $\sigma(R_{\text{min}}) = 2.7\% \times R_{\text{min}}$ and $\sigma(R_{\text{max}}) = 5.6\% \times R_{\text{max}}$. On the contrary, the measurement uncertainty on the effective dissociation constant of Fura was much higher [$\sigma(K_{d})$]...
20.0% × \(K_{\text{eff}}\). Consequently, the estimation of the calcium dynamics parameters is most affected by the measurement uncertainty on \(K_{\text{eff}}\). More precisely, considering that \(R_{\text{min}}\) and \(R_{\text{max}}\) are set to their experimental mean value in Eq. 2, it is straightforward to show that the calcium dynamics parameters \(\theta_{\text{Ca}}\), deduced from the ratiometric transformation (Eq. 8) are given by

\[
\bar{C}_a = C_{a0} \cdot \frac{K_{\text{eff}}}{K_{\text{eff}}^c} \quad (18a)
\]

\[
\Delta C_a = \Delta C_{a0} \cdot \frac{K_{\text{eff}}}{K_{\text{eff}}^c} \quad (18b)
\]

\[
\tau = \tau \quad (18c)
\]

where \(\theta_{\text{Ca}}\) stands for the true value of the calcium dynamics parameters. This means that an imprecision on the knowledge of \(K_{\text{eff}}\) will directly affect the value of the “amplitude” parameters \((C_{a0} \text{ and } \Delta C_{a})\), leading to substantial errors (from \(-38\) to \(151\%\); see Fig. 7). Unfortunately, the ratiometric method does not allow an estimation of the error on \(C_{a0}\) and \(\Delta C_{a}\). For the simulations of Fig. 3B, the CIs provided by the ratiometric fits were of the same order of magnitude as that obtained in Fig. 3A, but since the error on the calibrated parameters was not corrected, the true values of \(C_{a0}\) and \(\Delta C_{a}\) fell more seldom into these CIs.

To solve this problem, we then developed a new direct method, simply working on the original fluorescence measurements at both wavelengths, which did not imply any data ratioing. These fluorescence signals, acquired with a CCD camera, were modeled as realizations of Normal distributions with variance increasing linearly with the mean (see Eq. 5). More precisely, the parameters of the Normal distributions depended on the camera characteristics (gain \(G\) and SD of the readout noise \(\sigma_{\text{ro}}\)). The latter, estimated from “calibration” measurements (Fig. 2A), were found to be slightly larger than those stated in the technical description of the camera (\(G = 0.146\), compared with 0.133; \(\sigma_{\text{ro}} = 16-17\), compared with 14 photoelectrons).

Following Eq. 5, the variance of the fluorescence transients was time dependent. We then looked for a variable transformation stabilizing this variance and, inspired by the work of Bartlett (1936) and Anscombe (1948), we defined our square-root transformation as \(\sqrt{G \cdot \text{adu} + G \cdot \sigma_{\text{ro}}^2}\). This transformation stabilized the variance to \(G/4\) independently of the fluorescence intensity (see Eq. 12 and Fig. 2C), thus leading to the construction of meaningful CIs, as illustrated in Fig. 3A. Moreover, using approaches inspired by constrained linear regression, it was also possible to take into account the measurement uncertainty on the calibrated parameters (Eq. 13). This is particularly useful in real situations, where the actual value of these parameters can be reliably estimated simultaneously with the calcium dynamics and background fluorescence parameters (Fig. 3B).

In addition to the construction of meaningful confidence intervals, the use of the direct method in combination with the square-root transformation gives us stringent goodness-of-fit tests. As illustrated in Fig. 6, C and D, the iid assumption on the fluorescence noise can be tested using the autocorrelation function and a q–q plot. The adequation of a calcium dynamics model is assessed in a straightforward way from these plots.

The major advantage of the ratiometric transformation relies on the fact that the product \([B_1]_1\phi\) depending mainly on the light path through the tissue and the indicator total concentration within the cell—disappears when ratioing the fluorescence measurements at both wavelengths (Eq. 7), meaning that we do not have to estimate it. However, we showed here that the direct method allows a reliable estimation of this product: when \([B_1]_1\) is time independent, meaningful CIs can be constructed for \(\phi\) (Fig. 3). We also developed a three-wavelength protocol to deal with the more general case of a time-varying \([B_1]_1\) (see Methods and Appendix C). This protocol, derived from the isocoefficient method (Zhou and Neher 1993), uses the information provided by measurements performed at 340, 380, and 360 nm to estimate the isocoefficient \(\alpha\) and thus follow the time-dependent variations of \(\text{adu}_{340} + \alpha \cdot \text{adu}_{380}\), a signal proportional to \([B_1]_1\). As illustrated on experimental data (Figs. 4 and 5), variations of \([B_1]_1\) as small as 3% can lead to errors in the fits of the square roots of \(\text{adu}_{340}\) and \(\text{adu}_{380}\), which disappear when taking \(\alpha\) as a parameter (Fig. 6), thereby considerably improving the goodness of fit. In particular, the slope of the q–q plot was very close to the expected SD of the residuals theoretically given by the square-root transformation (Eq. 12). The accuracy of the direct fits directly depends on the CCD camera characteristics that are included in the square-root transformation. Here, these parameters have been found to be close to those stated in the technical description of the camera. Consequently, skipping the camera “calibration” step (Fig. 2) would not have jeopardized much the quality of our estimations.

The primary goal of this work was to develop a method for the accurate construction of confidence intervals for all calcium dynamics parameters, which we managed to do with the direct method. Our simulations focused on monoexponential decays, but similar results were obtained with biexponential decays and the method could be easily extended to more complex parametric decays. Interestingly, we also found that the ratiometric method provided meaningful confidence intervals for the calcium time constants. This theory-based and simulation-validated result can be further illustrated on the experimental data fitted in Fig. 6, for which we obtained the following values:

\[
\tau = 6.04 \pm 0.11 \text{ s with the ratiometric method and } 6.03 \pm 0.05 \text{ s with the direct methods. We emphasize that such accurate estimations would not have been obtained without the}
\]
development of a proper data-generation model for the fluorescence intensity and the CCD camera. Indeed, the ratiometric least-squares criterion (Eq. 9) includes estimates of the calcium concentration variance that are directly deduced from the data-generation model (see Appendix B and Eq. 5). Not considering such variations of the calcium concentration with time, and thus using a nonweighted least-squares criterion (as usually done in the literature), would have yielded underestimated CIs on the time constant. For instance, Monte Carlo simulations run with the parameters of Table 2 led to $N_S = 896$.

We emphasize that the direct method can be used with any parametric model of calcium dynamics. Indeed, this method is built on the basis of a fluorescence model that embeds a calcium model. The latter does not have to be the monoexponential model used here mainly for illustrative purposes. Biexponential models are already implemented in the CalciOMatic package and are readily usable. Differential-equation–based models will also be added in the future.

Since fits are quickly performed, the direct approach does not require working in a region of interest (ROI) pooling many pixels. Single-pixel transients can therefore be fitted to probe for calcium concentration gradients within a cell. In such approaches, the CIs on absolute calcium concentrations provided by the direct method are particularly useful.

Unlike the ratiometric method, the direct method does not directly use the background measurements to compute a ratio, but estimates the mean value of their Gaussian distribution. This estimation was shown to be accurate (Fig. 3), but relies on two major assumptions: the background distribution remains constant with time and is the same in the ROI and in the background region. Concerning the first assumption, we argue that a drift in the background fluorescence would decrease the goodness of fit and would be observed in the residuals. Thus we think that time-dependent variations of background—if present—were negligible. In our experiments, the background measurements were performed in the ROI before loading Fura into the cell, so that the second assumption should also be fulfilled. Unfortunately, the direct method does not yet allow us to rigorously test these two assumptions. Since a global offset in the background fluorescence would shift the estimations of the calcium baseline, estimating these parameters quantitatively is crucial. Such an attempt has been made by Chen et al. (2006). This interesting approach exploits the information of all pixels of the ROI instead of considering the ROI as a whole and thus does not require external background measurement. Unfortunately, no CI is associated with the estimated best values, which makes this approach incompatible with a quantitative parameter estimation, as done in our study. Future work will thus focus on the question of quantitative background fluorescence estimation.

The direct method has been described here in the context of organic buffers presenting spectral shifts in absorption and emitting at a single wavelength. This method can of course be applied to any kind of ratiometric measurements, for instance when dyes are excited at a single wavelength and emit differentially at two wavelengths. This is the case for the chemical dyes Indo-1 and Fluo-3 used in combination with Fura Red. It is also particularly well suited for the FRET-based genetically encoded calcium indicator TN-XXL (Mank et al. 2008). In such a context, using the direct method instead of computing ratios would highly benefit quantitative analysis of both in vitro and in vivo experiments.

In spite of their interest regarding the estimation of absolute calcium concentrations, ratiometric dyes like Fura-2 are not predominantly used. Instead, visible-light indicators such as Oregon Green BAPTA-1 (OGB-1), Calcium Green 1, or Fluo-3 are preferred. These dyes, well suited to two-photon imaging, generally present high brightness ratios ($\approx 10$), leading to large signal-to-noise ratios. However, since their absorption spectra are not shifted, these dyes do not allow a wavelength ratioing. Results reported in the literature are generally expressed in terms of $\Delta F/F$, which does not yield direct estimations of calcium dynamics. The only methodological work that aimed at determining calcium baseline and influx from $\Delta F/F$ signals has been done by Maravall et al. (2000). In that study, $C_{\text{calib}}$ and $\Delta Ca$ are expressed in terms of $\Delta F/F$, $\Delta F(F)_{\text{max}}$, $K_{\text{d}}$, and $R_F$. The latter are respectively the indicator dissociation constant and the fluorescence ratio between its calcium-bound and free forms and are evaluated from calibration experiments. Unfortunately, the measurement uncertainty on these quantities is not taken into account in the calculation of the calcium dynamics parameters, so that proper confidence intervals cannot be constructed. In a future study, we will address the question of the quantitative estimation of calcium dynamics from single-wavelength measurements.

In conclusion, the direct method provides reliable confidence intervals for all calcium dynamics parameters regardless of the calibrated parameter value, without requiring more data than those already needed to apply the ratiometric transformation. Moreover, it also allows one to handle time-dependent variations of $[B_i]$, for supplementary measurements at the isosbestic indicator wavelength. Thus we would like to encourage experimenters to analyze their data with this new direct method. The latter has been implemented in the open-source R environment, allowing free use by calcium imagers all over the world.

**Appendix A**

**Analytical expression of the SE of two estimators of $\tau$**

We consider a monoexponential model of fluorescence transient, given by $F(t, \tau) = F_0 + \Delta F \exp(-t/\tau)$, for which the values of $F_0$ and

### Table 2. Parameters used for the Monte Carlo simulations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\theta_{Ca}$</td>
<td>0.1 $\mu$M</td>
</tr>
<tr>
<td>$\Delta Ca$</td>
<td>0.25 $\mu$M</td>
</tr>
<tr>
<td>$\tau$</td>
<td>1.5 s</td>
</tr>
<tr>
<td>$\theta_{rpr}$</td>
<td>2</td>
</tr>
<tr>
<td>$s_{92,340}$</td>
<td>30 counts/(pixel-s)</td>
</tr>
<tr>
<td>$s_{92,180}$</td>
<td>80 counts/(pixel-s)</td>
</tr>
<tr>
<td>$\theta_{\text{rpr}}$</td>
<td></td>
</tr>
<tr>
<td>$R_{\text{min}}$</td>
<td>0.136 $\mu$M (SE = 0.00363)</td>
</tr>
<tr>
<td>$R_{\text{max}}$</td>
<td>2.701 $\mu$M (SE = 0.151)</td>
</tr>
<tr>
<td>$K_{\text{d}}$</td>
<td>3.637 $\mu$M (SE = 0.729)</td>
</tr>
<tr>
<td>$K_0$</td>
<td>0.583 $\mu$M (SE = 0.123)</td>
</tr>
<tr>
<td>Others</td>
<td></td>
</tr>
<tr>
<td>$[B_i]$</td>
<td>100 $\mu$M</td>
</tr>
<tr>
<td>$T_{92,340}$</td>
<td>0.015 s</td>
</tr>
<tr>
<td>$T_{92,180}$</td>
<td>0.006 s</td>
</tr>
<tr>
<td>$P, P_B$</td>
<td>200 pixels</td>
</tr>
<tr>
<td>$G$</td>
<td>1</td>
</tr>
<tr>
<td>$\sigma_{\text{inc}}$</td>
<td>0 photoelectron</td>
</tr>
<tr>
<td>$nb_{bg}$</td>
<td>1 background measurement</td>
</tr>
</tbody>
</table>
ΔF are known. Our goal is to estimate τ based on measurements \( \{y_i\}_{i=1, \ldots, N} \) which are modeled as realizations of Poisson distributions with parameters \( \{F(t_i, \tau)\}_{i=1, \ldots, N} \). For \( F(t_i, \tau) \geq 25 \), the latter can be well approximated by Gaussian distributions with variance equal to the mean, leading to

\[
y_i = F(t_i, \tau_0) + \sqrt{F(t_i, \tau_0)} \epsilon_i, \quad i = 1, \ldots, N \quad \text{for} \quad F(t_i, \tau_0) \geq 25
\]

(A1)

where \( \tau_0 \) is the true value of \( \tau \) and the \( \epsilon_i \) are independent and identically distributed from a standard normal distribution.

We consider two estimators of \( \tau \), obtained by minimizing the following residual sums of squares (RSS)

\[
\text{RSS}(\tau) = \sum_{i=1}^{N} [y_i - F(t_i, \tau)]^2 \quad (A2a)
\]

\[
\text{RSS}(\tau) = \sum_{i=1}^{N} [(y_i - F(t_i, \tau))]^2 \quad (A2b)
\]

The first estimator relies on the assumption that the noise variance is time independent (wrong noise model), whereas the second estimator takes into account the fact that the data arise from Poisson distributions, for which the square-root transformation stabilizes the variance (correct noise model; see METHODS for details about the square-root transformation). Here, we characterize both estimators of \( \tau \), in terms of expected value and SE.

For the wrong noise model, the optimal value of \( \tau \) (\( \hat{\tau} \)) is the one that minimizes \( \text{RSS}(\tau) \), i.e., the one for which the following expression is 0

\[
\hat{S}(\tau) = \frac{d \text{RSS}}{d\tau} (\tau) = -2 \sum_{i=1}^{N} [F(t_i, \tau) [y_i - F(t_i, \tau)]]
\]

(A3a)

where \( F_i \) is the first-order derivative of \( F \) with respect to \( \tau \). From Eq. A1, the expected value of the previous expression is

\[
E[\hat{S}(\tau)] = \frac{d \text{RSS}}{d\tau} (\tau) = -2 \sum_{i=1}^{N} [F(t_i, \tau) [F(t_i, \tau_0) - F(t_i, \tau)]]
\]

(A3b)

which equals 0 if and only if \( \tau = \hat{\tau} = \tau_0 \). Consequently, the estimator of \( \hat{\tau} \) is unbiased.

We now calculate the SE of the estimator of \( \hat{\tau} \). The first-order Taylor expansion of \( \hat{S}(\tau) \) writes

\[
\hat{S}(\tau) = \hat{S}(\tau_0 + \eta) = \hat{S}(\tau_0) + \frac{d\hat{S}}{d\tau} (\tau_0) \eta
\]

with

\[
\frac{d\hat{S}}{d\tau} (\tau_0) = -2 \sum_{i=1}^{N} [F_r(t_i, \tau_0)[y_i - F(t_i, \tau_0)] - F(t_i, \tau_0)^2]
\]

Thus

\[
\hat{S}(\tau) = \hat{S}(\tau_0) - 2 \sum_{i=1}^{N} [F_r(t_i, \tau_0)[y_i - F(t_i, \tau_0)] - F(t_i, \tau_0)^2] \eta
\]

\( \hat{\tau} = \tau_0 + \eta \) solves \( \hat{S}(\hat{\tau}) \), thus

\[
\eta = \frac{\hat{S}(\tau_0)}{2 \sum_{i=1}^{N} [F_r(t_i, \tau_0)[y_i - F(t_i, \tau_0)] - F(t_i, \tau_0)^2]}
\]

which, using Eq. A1, leads to

\[
\hat{\eta}((\epsilon_i)) = \frac{\hat{S}(\tau_0)}{2 \sum_{i=1}^{N} [F_r(t_i, \tau_0)\sqrt{F(t_i, \tau_0)} - F(t_i, \tau_0)^2]}
\]

By definition of \( \hat{S} \) (Eq. A3), we also have

\[
\hat{S}(\tau_0) = -2 \sum_{i=1}^{N} [F_r(t_i, \tau_0)\sqrt{F(t_i, \tau_0)} - F(t_i, \tau_0)^2]
\]

so that \( \hat{\eta}((\epsilon_i)) \) becomes

\[
\hat{\eta}((\epsilon_i)) = \frac{\sum_{i=1}^{N} [F_r(t_i, \tau_0)\sqrt{F(t_i, \tau_0)} - F(t_i, \tau_0)^2]}{\sum_{i=1}^{N} [F_r(t_i, \tau_0)\sqrt{F(t_i, \tau_0)} - F(t_i, \tau_0)^2]}
\]

(A4)

Since the \( \epsilon_i \) are independent, using the error propagation method leads to the following approximate variance of \( \hat{\eta} \)

\[
\sigma^2(\hat{\eta}) = \sum_{i=1}^{N} \left[ \frac{\partial \hat{\eta}}{\partial \epsilon_i} \right]^2 = \sum_{i=1}^{N} \left[ \frac{\partial \hat{\eta}}{\partial \epsilon_i} \right]^2 \epsilon_i = 0, \ldots, \epsilon_N = 0
\]

From Eq. A4, this expression becomes

\[
\sigma^2(\hat{\eta}) = \sum_{i=1}^{N} [F_r(t_i, \tau_0)\sqrt{F(t_i, \tau_0)} - F(t_i, \tau_0)^2]
\]

(A5)

The SE of the estimator of \( \hat{\tau} \) being the square root of the approximate variance of \( \hat{\eta} \).

Similar but slightly more tedious calculations show that the correct noise model gives an unbiased estimator of \( \tau \), \( \hat{\tau} \), the approximate variance of which is given by

\[
\sigma^2(\hat{\tau}) = \left[ \sum_{i=1}^{N} [F_r(t_i, \tau_0)\sqrt{F(t_i, \tau_0)} - F(t_i, \tau_0)^2] \right]^{-1}
\]

(A6)

APPENDIX B

Ratiometric transformation: estimation of the covariance matrix of the calcium signal

The covariance matrix of the free calcium concentration estimated with the ratiometric method is derived with a first-order Taylor expansion. Using Eqs. 6 and 8, and assuming the calibrated parameters exactly known, this expansion at two different time points \( t_i \) and \( t_j \) along the transient is given by

\[
\text{ca}_i = \text{Ca}(\text{adu}_{B,340}, \text{adu}_{B,380}, \text{adu}_{B,380})
\]

\[
= \text{Ca}(E(\text{adu}_{B,340}), E(\text{adu}_{B,380}), E(\text{adu}_{B,380}))
\]

\[
+ \left( \frac{\partial \text{Ca}}{\partial \text{adu}_{B,340}} \right) \sigma_{\text{adu}_{B,340}, E_{340}} + \left( \frac{\partial \text{Ca}}{\partial \text{adu}_{B,380}} \right) \sigma_{\text{adu}_{B,380}, E_{380}}
\]

\[
+ \left( \frac{\partial \text{Ca}}{\partial \text{adu}_{B,380}} \right) \sigma_{\text{adu}_{B,380}, E_{B,380}} + \left( \frac{\partial \text{Ca}}{\partial \text{adu}_{B,340}} \right) \sigma_{\text{adu}_{B,340}, E_{B,380}}
\]

(B1a)
The main assumption used to build confidence intervals in nonlinear regression is that the difference (residual) between the data (estimated free calcium) and their theoretical value has a multivariate Gaussian distribution. The first-order Taylor expansion gives the covariance matrix of this residual distribution. Nevertheless, it does not imply that a multivariate Gaussian distribution with the (asymptotically) correct mean value and the (asymptotically) correct covariance matrix is a “good enough” approximate of the true residuals distribution. This must be checked, for instance with simulation, on a case-specific basis. We performed simulations to test this hypothesis in a simple case where only two calcium levels were considered (Fig. B1). We found that the residuals distribution was close to a multivariate Gaussian when calibrated parameters were perfectly known (Fig. B1A), but differed much when these calibrated parameters were considered as random variables (Fig. B1B). An additional source of error is the fact that an estimation of the covariance matrix is used in practice, since the true one is unknown.

Moreover, we have observed that even in the case where our residuals distribution was a multivariate Gaussian, the log-likelihood profile of the regression model differed somewhat from a quadratic function. To build CIs, we thus did not use the Gaussian approximation of the likelihood, but considered the likelihood ratio statistics (Davison 2003).

**APPENDIX C**

**Estimation of the isocoefficient α with a three-wavelength protocol**

The isocoefficient method was introduced by Zhou and Neher (1993). In a recent review, Neher (2005) proposed to estimate α by minimizing the following sum of squares

\[
\sum_{j=1}^{J} \left( \frac{P_{i} \text{adu}_{340,j} - P_{i} \text{adu}_{380,j}}{T_{i,340}} + \frac{P_{i} \text{adu}_{380,j} - P_{i} \text{adu}_{340,j}}{T_{i,380}} - \beta \right)^2
\]

where \( J \) is the number of measurements performed during the transient. Since \( \beta \) is a constant, this method applies only to cases where \( B_{j} \) remains constant with time.

Here, we propose to extend this approach to situations where \( B_{j} \) varies with time, by using recordings at three wavelengths [i.e., the original 340 and 380 nm, and the Fura-2 isosbestic point (360 nm)]. Since \( \text{adu}_{360} \) is proportional to \( B_{j} \) (as \( \beta \) is, see Eq. 15b), one can...
ratiometric transformation (Eq. 8). In the 10,000 fluorescence transients were drawn from Poisson distributions, accord-
Unfortunately, this first approach provided only an estimation of
estimate contours), resulting in an experimental RSS close to a
parameters set to their experimental average value, the empirical distribution of
Ca2+] = 0.35 μM. In all, 10,000 fluorescence transients were drawn from Poisson distributions, according to the parameters given in Table 2. Values of \( \theta \) were then estimated by the ratiometric transformation (Eq. 8). In the left column, \( \theta \) for [Ca2+] = 0.35 μM are plotted against \( \theta \) for [Ca2+] = 0.10 μM, with the kernel-estimated 2-dimensional density function superimposed. In the right column, the quantiles of the empirical residual sum of squares (RSS) distribution are plotted against the quantiles of a \( \chi^2 \) distribution with 2 degrees of freedom (RSS theoretical distribution in a regression framework). A: with calibrated parameters set to their experimental average value, the empirical distribution of \( \theta \) was close to a bivariate Gaussian (note the elliptic shape of the density contours), resulting in an experimental RSS close to a \( \chi^2 \) distribution. B: on the contrary, when calibrated parameters were drawn from Gaussian distributions, \( \theta \) did not follow a bivariate Gaussian anymore (note the wedge shape of the density contours), and the RSS diverged from the \( \chi^2 \) distribution.

estimate \( \alpha \) and a new parameter \( \delta \) by minimizing the following modified sum of squares

\[
\sum_{j=1}^{J} \left( \frac{P^{-1}adu_{340j} - P^{-1}adu_{340}}{T_{\theta_{340}}} \right)^2 + \alpha \left( \frac{P^{-1}adu_{380j} - P^{-1}adu_{380}}{T_{\theta_{380}}} \right)^2
+ \delta \left( \frac{P^{-1}adu_{360j} - P^{-1}adu_{360}}{T_{\theta_{360}}} \right)^2 (C2)
\]

Unfortunately, this first approach provided only an estimation of \( \bar{\theta} \), but not SE \( \sigma(\bar{\theta}) \) because the variances of \( adu_{340}, \alpha adu_{380}, \) and \( \delta adu_{360} \) were not uniform (and unpredictable, since \( \alpha \) and \( \delta \) are unknown before the fit). To face this difficulty, we used a Monte Carlo approach. Based on the smoothed versions of \( adu_{340}, adu_{380}, \) and \( adu_{360} \) we generated 1,000 noisy data sets according to Eq. 5. Then, we minimized Eq. C2, which provided 1,000 fitted values of \( \alpha \) (and of \( \delta \) as well). The mean and SD of this sample were then calculated to give \( \bar{\alpha} \) and \( \sigma(\bar{\alpha}) \).

FIG. B1. Influence of the measurement uncertainty of the calibrated parameters on the distribution of the calcium concentration estimated by the ratiometric method. Two fluorescence levels were considered, corresponding to [Ca2+] = 0.10 μM and [Ca2+] = 0.35 μM. In all, 10,000 fluorescence transients were drawn from Poisson distributions, according to the parameters given in Table 2. Values of \( \theta \) were then estimated by the ratiometric transformation (Eq. 8). In the left column, \( \theta \) for [Ca2+] = 0.35 μM are plotted against \( \theta \) for [Ca2+] = 0.10 μM, with the kernel-estimated 2-dimensional density function superimposed. In the right column, the quantiles of the empirical residual sum of squares (RSS) distribution are plotted against the quantiles of a \( \chi^2 \) distribution with 2 degrees of freedom (RSS theoretical distribution in a regression framework). A: with calibrated parameters set to their experimental average value, the empirical distribution of \( \theta \) was close to a bivariate Gaussian (note the elliptic shape of the density contours), resulting in an experimental RSS close to a \( \chi^2 \) distribution. B: on the contrary, when calibrated parameters were drawn from Gaussian distributions, \( \theta \) did not follow a bivariate Gaussian anymore (note the wedge shape of the density contours), and the RSS diverged from the \( \chi^2 \) distribution.

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