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Fast algorithms for the analysis of single and double exponential decay curves with a background term.

Application to time-resolved imaging microscopy

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Abstract. Computer programs have been developed to determine decay time constants from a temporal sequence of digitized images with decaying intensities characterized by either single or double exponentials plus a constant background term. The very fast algorithms are evaluated at every pixel position. A non-iterative Prony-like method provides high quality initial estimates that are used for the subsequent non-linear least-squares procedure in which the normal equations are solved directly. Error analysis routines enable a pixel-by-pixel estimation of the quality of the experimental data. The stand-alone programs were fully integrated into a commercial image-processing environment (SCIL-Image) for a convenient and optimal display of the decay analysis. The features of the programs are illustrated by the analysis of simulated image data. With current workstations, the fitting routines (including reading of data, initial estimate and error analysis) require 0.13 ms/pixel using the single exponential algorithm applied to 50 time points, and 1.37 ms/pixel for 100 time points and the double exponential algorithm. The programs are of general applicability and have been used to analyse data from time-resolved fluorescence, phosphorescence, and photobleaching-based microscopy. Two examples of the latter case are shown, illustrating the utility of the programs for the quantitative evaluation of spatially resolved fluorescence resonance energy transfer (FRET) and for generating contrast by allocating specific cellular structures to particular decay components in a fluorescence image.

Keywords: fluorescence microscopy, fluorescence resonance energy transfer, FRET, image processing, photobleaching

1. Introduction

Recent innovations in digital imaging microscopy include fluorescence lifetime imaging microscopy (FLIM) [1–15], delayed luminescence or phosphorescence imaging microscopy [16–18] and photobleaching imaging microscopy

[19–24]. These techniques enable the study of the excited state-photodynamics of fluorophores in the complex environment of a biological specimen with the high resolution and sensitivity of the fluorescence microscope. In addition to achieving novel ways for generating contrast in an image, the ultimate goal of the above techniques is to extract an image of the time constants (or lifetimes) that describe the excited state photophysics of the probes in the specimen. These time constants are generally inde-

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pendent of fluorescence intensity and provide the means for an accurate quantitation of the amount and microenvironment of fluorescent probes [4]. For the calculation of time constants from time-resolved imaging microscopy (TRIM) data, appropriate strategies for data analysis and image processing are essential, with particular emphasis on efficiency and robustness. Such post-acquisition processing has been described in a previous publication [25] concerned with the frequency-domain implementation of FLIM [3–5, 7, 9, 13, 26]. The algorithms for time-domain FLIM and delayed luminescence microscopy are much more involved inasmuch as they depend on non-linear parameter estimation [27] applied to a large body of data. In time-domain FLIM, a fast and simple ratio imaging procedure has been used to reconstruct the fluorescence lifetime image [2, 6, 8, 10–12, 14, 28]. However, one disadvantage of this procedure is that only an average lifetime image that is very dependent on the selection of the time-windows (in the case of multicomponent systems) is generated. In addition, information on the quality of the data is absent.

In this paper we present fast algorithms for calculating single and double exponential decay curves with a background term from a series of images which can be applied to time-domain FLIM, phosphorescence microscopy and photobleaching microscopy. The programs described here have evolved over the past six years starting from an original algorithm [29] based on a direct evaluation of the normal equations arising from the least squares solution of a single exponential fit [30]. Although the underlying algorithms were not presented in detail, this technique has been applied to the evaluation of data obtained in phosphorescence microscopy [31, 32] and photobleaching microscopy [21, 24, 29, 33–37].

2. Fit procedure

The algorithms described below are devised to fit the digitized intensities of a temporal sequence of images obtained by time-resolved imaging optical microscopy, to a single or double exponential decay. The digital images can be acquired either directly by a CCD-camera (as in photobleaching microscopy [21, 24, 35, 36]), by a combination of light choppers and a CCD-camera as in phosphorescence microscopy [18] by photomultiplier tubes (in the case of confocal time-domain FLIM [38]), or by a combination of an image intensifier with a CCD-camera (as in time-domain FLIM ([11])). For each detection scheme, specific noise factors are introduced, depending on the gain, the analog to digital conversion factor and the digitization errors, as well as dark currents and readout noise [39, 40]. Other noise factors influencing the entire images may be introduced by lamp or laser intensity fluctuations or by image intensifier gating uncertainties. Hence, the overall distorting effects may differ from one system to the other, but in general will to a large extent

be governed by counting statistics (Poisson noise). In the fit procedures the difference between observed and calculated data is minimized according to an unweighted least squares procedure, and no model for the uncertainties in the observed data is introduced. Inasmuch as these fit procedures do not account for systematic deviations between experimental and calculated data, the uncertainties in the fitted parameters are estimated assuming the absence of such effects.

Analysis of single-exponential decays in sequences of images has been successfully implemented by others [30, 41] although the methods of data reduction were laborious and time consuming. The algorithm described here is inherently very efficient due to a combination of (i) a fast (non-iterative) and very accurate estimation of initial parameters based on the Prony method [42], (ii) bypassing the need for calculating summations of exponentials, and (iii) elimination of linear parameters in the numerical minimization of the least squares procedure, enabling (iv) a direct solution of normal equations without time-consuming iterations on parameters as in conventional Levenberg–Marquardt techniques.

2.1. Single exponential fit

Since the image sequences are necessarily acquired with a finite integration time (= camera exposure time), the fitted intensity data for each pixel i ($y_{c,i}$) represent an integration over time (δt) of a single exponential decay curve with decay time constant τ , pre-exponential factor α and a constant background term c according to the following equation:

$$y_{c,i} = \frac{\int_{t_i}^{t_i+\delta t} \{\alpha \exp(-t/\tau) + c\} dt}{\int_{t_i}^{t_i+\delta t} dt} = \alpha' \exp(-t_i/\tau) + c \quad (1)$$

$$\alpha' = \frac{\alpha \tau}{\delta t} (1 - \exp(-\delta t/\tau)).$$

If the images are taken at equidistant time-points separated by a time interval Δt , equation (1) can be transformed into the form given by equation (2). This has major advantages in view of the calculation time, as will be outlined below.

$$y_{c,i} = \alpha' \exp\left(-\frac{\Delta t}{\tau}(i-1)\right) + c = ap^i + c \quad (2)$$

$$p = \exp(-\Delta t/\tau), \quad a = \alpha'/p.$$

As described above for a fit criterion, one seeks to minimize the sum of the squared differences (ϵ_i) between the observed decay curve ($y_{o,i}$) and the single exponential (equation (2)).

$$\begin{aligned} \text{SSQ} &= \sum_{i=1}^n \epsilon_i^2 = \sum_{i=1}^n \{y_{c,i} - y_{o,i}\}^2 \\ &= \sum_{i=1}^n \{ap^i + c - y_{o,i}\}^2 \rightarrow \text{minimal}. \end{aligned} \quad (3)$$

The procedure for finding the global minimum of SSQ is analogous to one described elsewhere [30], in which the normal equations (the partial derivatives of equation (3) with respect to a , p and c set to zero) are solved directly. These equations are given below.

$$a \sum_{i=1}^n p^{2i} + c \sum_{i=1}^n p^i - \sum_{i=1}^n y_{0,i} p^i = 0 \quad (4a)$$

$$a \sum_{i=1}^n i p^{2i} + c \sum_{i=1}^n i p^i - \sum_{i=1}^n i y_{0,i} p^i = F(p) = 0 \quad (4b)$$

$$a \sum_{i=1}^n p^i + cn - \sum_{i=1}^n y_{0,i} = 0. \quad (4c)$$

From the first and the last normal equations the linear parameters a and c can be expressed as functions of the non-linear parameter p according to equations (5) and (6):

$$a(p) = \frac{n \sum_{i=1}^n y_{0,i} p^i - \left(\sum_{i=1}^n y_{0,i} \right) \left(\sum_{i=1}^n p^i \right)}{n \sum_{i=1}^n p^{2i} - \left(\sum_{i=1}^n p^i \right)^2} = \frac{F1(p)}{F2(p)} \quad (5)$$

$$c(p) = \frac{\left(\sum_{i=1}^n y_{0,i} \right) \left(\sum_{i=1}^n p^{2i} \right) - \left(\sum_{i=1}^n y_{0,i} p^i \right) \left(\sum_{i=1}^n p^i \right)}{n \sum_{i=1}^n p^{2i} - \left(\sum_{i=1}^n p^i \right)^2} = \frac{F3(p)}{F2(p)}. \quad (6)$$

The separation of linear and non-linear parameters has been described before [43]. In principle, one can substitute equations (5) and (6) into equation (3) and use Marquardt's method [44] to minimize the SSQ for the single non-linear parameter. However, we avoid the time consuming non-linear regression procedure by directly evaluating equation (4b) after substituting $a(p)$ with equation (5) and $c(p)$ with equation (6). This yields a single non-linear (in principle polynomial) function $F(p)$ (equation (4b)) which can be solved using the Newton-Raphson technique [44]. We chose this technique as it converges very fast close to the solution by essentially doubling the number of correct digits at each step [42]. The next improved estimate of p (p_{new}) is produced according to

$$p_{\text{new}} = p - F(p) / (\partial F(p) / \partial p). \quad (7)$$

The partial derivative in equation (7) is given by

$$\begin{aligned} \left(\frac{\partial F(p)}{\partial p} \right) &= \left(\frac{\partial a(p)}{\partial p} \right) \sum_{i=1}^n i p^{2i} + \left(\frac{\partial c(p)}{\partial p} \right) \sum_{i=1}^n i p^i \\ &+ \frac{2a(p)}{p} \sum_{i=1}^n i^2 p^{2i} + \frac{c(p)}{p} \sum_{i=1}^n i^2 p^i - \frac{1}{p} \sum_{i=1}^n y_{0,i} i^2 p^i \end{aligned} \quad (8)$$

with

$$\left(\frac{\partial a(p)}{\partial p} \right) = \frac{F_2(p) (\partial F_1(p) / \partial p) - F_1(p) (\partial F_2(p) / \partial p)}{(F_2(p))^2} \quad (8a)$$

$$\left(\frac{\partial c(p)}{\partial p} \right) = \frac{F_2(p) (\partial F_3(p) / \partial p) - F_3(p) (\partial F_2(p) / \partial p)}{(F_2(p))^2} \quad (8b)$$

and

$$\left(\frac{\partial F_1(p)}{\partial p} \right) = \frac{1}{p} \left\{ n \sum_{i=1}^n y_{0,i} i p^i - \left(\sum_{i=1}^n y_{0,i} \right) \left(\sum_{i=1}^n i p^i \right) \right\} \quad (8c)$$

$$\left(\frac{\partial F_2(p)}{\partial p} \right) = \frac{2}{p} \left\{ n \sum_{i=1}^n i p^{2i} - \left(\sum_{i=1}^n p^i \right) \left(\sum_{i=1}^n i p^i \right) \right\} \quad (8d)$$

$$\begin{aligned} \left(\frac{\partial F_3(p)}{\partial p} \right) &= \frac{1}{p} \left\{ 2 \left(\sum_{i=1}^n y_{0,i} \right) \left(\sum_{i=1}^n i p^{2i} \right) n \sum_{i=1}^n i p^{2i} \right. \\ &\quad \left. - \left(\sum_{i=1}^n i p^i \right) \left(\sum_{i=1}^n y_{0,i} p^i \right) - \left(\sum_{i=1}^n p^i \right) \left(\sum_{i=1}^n y_{0,i} i p^i \right) \right\}. \end{aligned} \quad (8e)$$

When p no longer changes significantly (we use the relative criterion $|\ln(p_{\text{new}}/p)| < \varepsilon$; $\varepsilon = 0.0001$) the global minimum of SSQ has been found. The values of a , p and c for which SSQ is minimal are designated $\bar{a}$, $\bar{p}$ and $\bar{c}$, respectively. The decay time constant τ and the pre-exponential factor α (corrected for integration time) can be calculated simply from $\bar{p}$ and $\bar{a}$:

$$\tau = \frac{-\Delta t}{\ln \bar{p}} \quad \alpha = \bar{a} \bar{p} \frac{\ln \bar{p}^\sigma}{\bar{p}^\sigma - 1} \quad \sigma = \frac{\delta t}{\Delta t}. \quad (9)$$

The limiting value for α (if σ approaches zero) equals $\bar{a} \bar{p}$.

The reason for fitting p and not τ is that most of the summations can be computed without time consuming loops which must be re-evaluated each iteration for all the data points. The summations over p can be evaluated analytically according to equation (10) [45].

$$\sum_{i=1}^n p^i = \frac{p}{1-p} (1-p^n) \quad (10a)$$

$$\sum_{i=1}^n i p^i = \frac{p}{(1-p)^2} \{1 - [n(1-p) + 1] p^n\} \quad (10b)$$

$$\sum_{i=1}^n i^2 p^i = \frac{p}{(1-p)^3} \{1 + p - [(n(1-p) + 1)^2 + p] p^n\}. \quad (10c)$$

The summations $\sum_{i=1}^n p^{2i}$, $\sum_{i=1}^n i p^{2i}$ and $\sum_{i=1}^n i^2 p^{2i}$ are obtained from equations (10a), (10b) and (10c), respectively, by substitution of p^2 for p . The other summations including the observed data ($y_{0,i}$) have to be calculated in a loop, which in fact consumes most of the time in the fit procedure, but still is fast since no exponentials have to be computed.

2.1.1. Initial estimate. A prerequisite for the iterative procedure described above is a good initial estimate of p . This we obtain by implementing a Prony-like method of exponential approximation. Like our fit procedure, the Prony method focuses on first finding the exponents and then the linear coefficients, and only applies to equally time-spaced data. Prony reduces the exponential approximation problem to the solution of an n th order characteristic polynomial equation (n is the number of exponents) [46]. We employ his strategy but instead of evaluating the homogeneous linear difference equation to generate the characteristic polynomial equation, we carry out a numerical integration (or in our case summation) of the actual data as described elsewhere for convoluted decay analysis [47, 48]. This strategy effectively smoothes the data, yielding more accurate estimates. The numerical summations of the actual data ($y_{o,i}$) and calculated (or expected) data ($y_{c,i}$) in time are given in the following equation:

$$Y_{o,i} = \sum_{j=1}^i y_{o,j} \quad (11a)$$

$$Y_{c,i} = \sum_{j=1}^i y_{c,j} = a \sum_{j=1}^i p^j + \sum_{j=1}^i c = \frac{ap}{p-1} p^i + ci + \frac{ap}{1-p}. \quad (11b)$$

The linear set of n equations with respect to α_1, α_2 and α_3 is given in equation (12a). Substitution of $Y_{c,i}$ for $Y_{o,i}$ and $y_{c,i}$ for $y_{o,i}$ leads to

$$\alpha_1 Y_{o,i} + \alpha_2 i + \alpha_3 = y_{o,i} \quad (i = 1, 2, 3 \dots n) \quad (12a)$$

$$ap^i \left(1 + \alpha_1 \frac{p}{1-p}\right) + i(c - \alpha_2) + \left(c - \frac{\alpha_1 ap}{1-p} - \alpha_3\right) = 0. \quad (12b)$$

Equation (12b) can be satisfied for all n values of i only if the three terms between the brackets are zero. This directly yields the characteristic (first order) polynomial equation $(1 + \alpha_1 p/(1-p)) = 0$, a condition satisfied when $p = (1 - \alpha_1)^{-1}$, which is then the desired initial value for p . It also follows that $c = \alpha_2$ and $a = \alpha_3 - \alpha_2$. The values of α_1, α_2 and α_3 best describing equation (12a) for all n values of i are found by minimizing the sum of squares SSQ of equation (12a) for α_1, α_2 and α_3 .

$$\text{SSQ} = \sum_{i=1}^n (y_{o,i} - \alpha_1 Y_{o,i} - \alpha_2 i - \alpha_3)^2 \rightarrow \text{minimal}. \quad (13)$$

The solution of this linear least squares problem is [44, 49]:

$$\begin{pmatrix} \alpha_1 \\ \alpha_2 \\ \alpha_3 \end{pmatrix} = \begin{bmatrix} \sum_{i=1}^n Y_{o,i}^2 & \sum_{i=1}^n i Y_{o,i} & \sum_{i=1}^n Y_{o,i} \\ \sum_{i=1}^n i Y_{o,i} & \sum_{i=1}^n i^2 & \sum_{i=1}^n i \\ \sum_{i=1}^n Y_{o,i} & \sum_{i=1}^n i & n \end{bmatrix}^{-1} \begin{pmatrix} \sum_{i=1}^n Y_{o,i} y_{o,i} \\ \sum_{i=1}^n i y_{o,i} \\ \sum_{i=1}^n y_{o,i} \end{pmatrix}. \quad (14)$$

This initial estimation procedure has proven to be very fast (only one step, no iteration, no initial guess by the user required), robust and accurate as discussed below in section 5.2.

2.1.2. Error analysis. To estimate the quality of the fit, two statistical parameters are calculated for each pixel: the residual (equation (15)) and the correlation coefficient (equation (16)) in the minimum of the sum of squares SSQ(min) (defined below in equation (21) [44]).

$$\text{Res} = \sqrt{\frac{\text{SSQ}(\min)}{\sum_{i=1}^n y_{o,i}^2}} \quad (15)$$

$$\text{Corr} = \sqrt{1 - \frac{\text{SSQ}(\min)}{\sum_{i=1}^n y_{o,i}^2 - \frac{1}{n} \left(\sum_{i=1}^n y_{o,i}\right)^2}}. \quad (16)$$

Error analysis was performed with a linearization technique [44], which can also serve to minimize the SSQ (see also section 8.7 in [50]). This assumes that the fitted function can be approximated by a first order Taylor expansion according to

$$y_{c,i} \approx \bar{a} \bar{p}^i + \bar{c} + (a - \bar{a}) \left(\frac{\partial y_{c,i}}{\partial a} \right)_{\bar{a}, \bar{p}, \bar{c}} + (p - \bar{p}) \left(\frac{\partial y_{c,i}}{\partial p} \right)_{\bar{a}, \bar{p}, \bar{c}} + (c - \bar{c}) \left(\frac{\partial y_{c,i}}{\partial c} \right)_{\bar{a}, \bar{p}, \bar{c}}. \quad (17)$$

The sum of squares is reformulated into the following equation in which only a, p and c are ‘variables’, whereas $\bar{a}, \bar{p}$ and $\bar{c}$ are ‘constants’:

$$\text{SSQ} = \sum_{i=1}^n \left\{ a [\bar{p}^i] + p \left[\frac{\bar{a}}{\bar{p}} i \bar{p}^i \right] + c - [y_{o,i} + \bar{a} i \bar{p}^i] \right\}^2. \quad (18)$$

The variance/covariance matrix is obtained via a matrix inversion procedure [44, 49]. The diagonal elements correspond to the estimated variances of the parameters a, p and c , whereas the off-diagonal elements correspond to the estimated covariances between the three parameters. The elements of the matrix β to be inverted are [44, 49]

$$\beta_{k,l} = \sum_{i=1}^n \left(\frac{\partial y_{c,i}}{\partial \theta_k} \right)_{\bar{\theta}} \left(\frac{\partial y_{c,i}}{\partial \theta_l} \right)_{\bar{\theta}} \quad \bar{\theta} = \begin{pmatrix} \bar{a} \\ \bar{p} \\ \bar{c} \end{pmatrix}. \quad (19)$$

The inverse matrix β multiplied by SSQ(minimum)/($n-3$) yields the variance/covariance matrix $\mathbf{M}$:

$$\mathbf{M} = \frac{\text{SSQ}(\min)}{n-3} \begin{bmatrix} \sum_{i=1}^n \bar{p}^{2i} & \frac{\bar{a}}{\bar{p}} \sum_{i=1}^n i \bar{p}^{2i} & \sum_{i=1}^n \bar{p}^i \\ \frac{\bar{a}}{\bar{p}} \sum_{i=1}^n i \bar{p}^{2i} & \left(\frac{\bar{a}}{\bar{p}} \right)^2 \sum_{i=1}^n i^2 \bar{p}^{2i} & \frac{\bar{a}}{\bar{p}} \sum_{i=1}^n i \bar{p}^i \\ \sum_{i=1}^n \bar{p}^i & \frac{\bar{a}}{\bar{p}} \sum_{i=1}^n i \bar{p}^i & n \end{bmatrix}^{-1} \quad (20)$$

with

$$\text{SSQ}(\min) = \bar{a}^2 \sum_{i=1}^n \bar{p}^{2i} + n\bar{c}^2 + \sum_{i=1}^n y_{o,i}^2 + 2 \left(\bar{a}\bar{c} \sum_{i=1}^n \bar{p}^i - \bar{a} \sum_{i=1}^n y_{o,i} \bar{p}^i - \bar{c} \sum_{i=1}^n y_{o,i} \right). \quad (21)$$

The summations in the matrix are already calculated during the fit procedure. Thus, the error analysis is much faster than the fit procedure (see below).

The error of any function of the three parameters $\bar{a}$, $\bar{p}$ and $\bar{c}$ ($f(\bar{a}, \bar{p}, \bar{c})$) may be estimated according to

$$s_f^2 = \sum_{k=1}^3 \sum_{\ell=1}^3 m_{k,\ell} \left(\frac{\partial f}{\partial \theta_k} \right)_{\bar{\theta}} \left(\frac{\partial f}{\partial \theta_\ell} \right)_{\bar{\theta}} \quad \bar{\theta} = \begin{pmatrix} \bar{a} \\ \bar{p} \\ \bar{c} \end{pmatrix}. \quad (22)$$

In this formula $m_{k,\ell}$ are the elements of matrix $\mathbf{M}$, hence $m_{i,i}$ is the estimated variance of parameter θ_i and $m_{i,j}$ is the estimated covariance between parameters θ_i and θ_j . From this equation the estimated errors in the parameters α , τ and c can be calculated according to equations (23), (24) and (25), respectively:

$$s(\alpha) = \sqrt{\left(\frac{\partial \alpha}{\partial \bar{a}} \right)^2 m_{11} + 2 \left(\frac{\partial \alpha}{\partial \bar{a}} \right) \left(\frac{\partial \alpha}{\partial \bar{p}} \right) m_{12} + \left(\frac{\partial \alpha}{\partial \bar{p}} \right)^2 m_{22}} \quad (23a)$$

with

$$\left(\frac{\partial \alpha}{\partial \bar{p}} \right) = \sigma \bar{a} \frac{(1 + \ln \bar{p})(\bar{p}^\sigma - 1) - \sigma \bar{p}^\sigma \ln \bar{p}}{(\bar{p}^\sigma - 1)^2}$$

and

$$\left(\frac{\partial \alpha}{\partial \bar{a}} \right) = \frac{\bar{p} \ln \bar{p}^\sigma}{\bar{p}^\sigma - 1}. \quad (23b)$$

If $\sigma = 0$, equation (23a) assumes the form

$$s(\alpha) = \sqrt{\bar{p}^2 m_{11} + 2\bar{a} \bar{p} m_{12} + \bar{a}^2 m_{22}}. \quad (23c)$$

Furthermore,

$$s(\tau) = \left| \left(\frac{\partial \tau}{\partial \bar{p}} \right) \right| \sqrt{m_{22}} = \frac{\Delta t}{\bar{p} \ln^2 \bar{p}} \sqrt{m_{22}} \quad (24)$$

$$s(c) = |m_{33}|. \quad (25)$$

2.2. Double exponential fit procedure

The double exponential fit procedure is analogous to the single exponential procedure. The observed decay is assumed to fit the description

$$y_{c,i} = \frac{\int_{t_i}^{t_i+\delta t} \{\alpha \exp(-t/\tau_1) + \beta \exp(-t/\tau_2) + c\} dt}{\int_{t_i}^{t_i+\delta t} dt} = ap^i + bq^i + c$$

$$a = \frac{\alpha \tau_1}{\delta t p} (1 - \exp(-\delta t/\tau_1)) \quad p = \exp(-\Delta t/\tau_1)$$

$$b = \frac{\beta \tau_2}{\delta t q} (1 - \exp(-\delta t/\tau_2)) \quad q = \exp(-\Delta t/\tau_2). \quad (26)$$

The sum of squares (SSQ) to be minimized is given by

$$\text{SSQ} = \sum_{i=1}^n \varepsilon_i^2 = \sum_{i=1}^n \{y_{c,i} - y_{o,i}\}^2 = \sum_{i=1}^n \{ap^i + bq^i + c - y_{o,i}\}^2. \quad (27)$$

Five normal equations are produced:

$$\begin{aligned} a \sum_{i=1}^n p^{2i} + b \sum_{i=1}^n (pq)^i + c \sum_{i=1}^n p^i - \sum_{i=1}^n y_{o,i} p^i &= 0 \\ a \sum_{i=1}^n i p^{2i} + b \sum_{i=1}^n i (pq)^i + c \sum_{i=1}^n i p^i - \sum_{i=1}^n i y_{o,i} p^i &= 0 \\ a \sum_{i=1}^n (pq)^i + b \sum_{i=1}^n q^{2i} + c \sum_{i=1}^n q^i - \sum_{i=1}^n y_{o,i} q^i &= 0 \\ a \sum_{i=1}^n i (pq)^i + b \sum_{i=1}^n i q^{2i} + c \sum_{i=1}^n i q^i - \sum_{i=1}^n i y_{o,i} q^i &= 0 \\ a \sum_{i=1}^n p^i + b \sum_{i=1}^n q^i + cn - \sum_{i=1}^n y_{o,i} &= 0. \end{aligned} \quad (28)$$

The first, third and fifth normal equations are linear with respect to a , b and c and can be solved directly via matrix inversion:

$$\begin{pmatrix} a(p, q) \\ b(p, q) \\ c(p, q) \end{pmatrix} = \begin{pmatrix} \sum_{i=1}^n p^{2i} & \sum_{i=1}^n (pq)^i & \sum_{i=1}^n p^i \\ \sum_{i=1}^n i p^{2i} & \sum_{i=1}^n i (pq)^i & \sum_{i=1}^n i p^i \\ \sum_{i=1}^n p^i & \sum_{i=1}^n q^i & n \end{pmatrix}^{-1} \begin{pmatrix} \sum_{i=1}^n y_{o,i} p^i \\ \sum_{i=1}^n i y_{o,i} p^i \\ \sum_{i=1}^n y_{o,i} \end{pmatrix}. \quad (29)$$

Here use is also made of the simplification of the summations (see equation (10)) and, if necessary, p^2 , q , pq , or q^2 are substituted for p . The parameters $a(p, q)$, $b(p, q)$ and $c(p, q)$ can be combined with the second and fourth normal equations yielding two non-linear functions, $F(p, q)$ and $G(p, q)$, with the two unknowns, p and q :

$$\begin{aligned} F(p, q) &= a(p, q) \sum_{i=1}^n i p^{2i} + b(p, q) \sum_{i=1}^n i (pq)^i \\ &\quad + c(p, q) \sum_{i=1}^n i p^i - \sum_{i=1}^n i y_{o,i} p^i = 0 \\ G(p, q) &= a(p, q) \sum_{i=1}^n i (pq)^i + b(p, q) \sum_{i=1}^n i q^{2i} \\ &\quad + c(p, q) \sum_{i=1}^n i q^i - \sum_{i=1}^n i y_{o,i} q^i = 0. \end{aligned} \quad (30)$$

In the minimization procedure, the fastest decaying component is fixed temporarily. Since the equations are symmetric with respect to p and q one can, for example,

assume that $q < p$ and hence that q is (temporarily) fixed. The next estimate for p (p_{new}) is found from

$$p_{\text{new}} = p - F(p, q) \left/ \left(\frac{\partial F(p, q)}{\partial p} \right) \right. \quad (31)$$

In order to avoid excessively large steps or blowup during the optimization of p , two safety precautions are built into the fit procedure. First, a bisectioning step is included if $F(p_{\text{new}}, q)/F(p, q) < -1$. Then the next better estimate of p is found from $p_{\text{next}} = (p_{\text{new}} + p)/2$. Secondly, if $0.625 > \ln(p_{\text{new}})/\ln(p) > 1.6$ the next estimate of p is found from $p_{\text{next}} = p^{1.6}$ if $F(p, q) > 0$ or $p_{\text{next}} = p^{0.625}$ if $F(p, q) < 0$. The next iteration is started using equations (29–31) until p does not change significantly ($|\ln(p_{\text{new}}/p)| < \varepsilon$; $\varepsilon = 0.0001$). In this situation, four of the five parameters are optimized. Finally, the fifth parameter q is optimized by using a two dimensional Newton–Raphson technique. Equation (30) can be rewritten as

$$\begin{aligned} F(p_{\text{new}}, q_{\text{new}}) &\approx F(p, q) + \Delta p \frac{\partial F(p, q)}{\partial p} \\ &\quad + \Delta q \frac{\partial F(p, q)}{\partial q} \\ G(p_{\text{new}}, q_{\text{new}}) &\approx G(p, q) + \Delta p \frac{\partial G(p, q)}{\partial p} \\ &\quad + \Delta q \frac{\partial G(p, q)}{\partial q} \end{aligned} \quad (32)$$

with $\Delta p = p_{\text{new}} - p$ and $\Delta q = q_{\text{new}} - q$. The next best estimates for both p and q are found from

$$\begin{pmatrix} p_{\text{new}} \\ q_{\text{new}} \end{pmatrix} = \begin{pmatrix} p \\ q \end{pmatrix} - \left[\frac{\partial F(p, q)}{\partial p} \quad \frac{\partial F(p, q)}{\partial q} \right]^{-1} \begin{pmatrix} F(p, q) \\ G(p, q) \end{pmatrix}. \quad (33)$$

The partial derivatives in the equations above were derived analytically but are not shown here. A safety precaution was built in: if $0.9 > \ln(p_{\text{new}})/\ln(p) > 1.1$ or $0.9 > \ln(q_{\text{new}})/\ln(q) > 1.1$, the last estimate for p is used ($p_{\text{new}} = p$), and the next estimate for q (q_{next}) is found from $q_{\text{next}} = q^{1.1}$ if $G(p, q) > 0$ or $q_{\text{next}} = q^{0.9}$ if $G(p, q) < 0$. The next iteration is initiated by fixing the new value for q and optimizing p as described above. If neither p nor q changes significantly ($|\ln(p_{\text{new}}/p)| < \varepsilon$; $\varepsilon = 0.0001$ and $|\ln(q_{\text{new}}/q)| < \varepsilon$; $\varepsilon = 0.0001$) the minimum has been found.

The decay time constants τ_1 and τ_2 with corresponding pre-exponential factors α and β (corrected for integration time) are calculated from the values of a , p , b and q for which SSQ is minimal ($\bar{a}$, $\bar{p}$, $\bar{b}$ and $\bar{q}$, respectively) according to

$$\begin{aligned} \alpha &= \bar{a} \bar{p} \frac{\ln \bar{p}^\sigma}{\bar{p}^\sigma - 1} & \tau_1 &= -\frac{\Delta t}{\ln \bar{p}} & \beta &= \bar{b} \bar{q} \frac{\ln \bar{q}^\sigma}{\bar{q}^\sigma - 1} \\ \tau_2 &= -\frac{\Delta t}{\ln \bar{q}} & \sigma &= \frac{\delta t}{\Delta t}. \end{aligned} \quad (34)$$

The limiting values for α and β when σ approaches zero are $\bar{a} \bar{p}$ and $\bar{b} \bar{q}$, respectively.

2.2.1. Initial estimate. The initial estimate is found by a similar procedure as described for the single exponential case. Here the first and second order numerical integrals are considered.

$$Y_{0,i} = \sum_{j=1}^i y_{0,j} \quad (35a)$$

$$\begin{aligned} Y_{c,i} &= \sum_{j=1}^i y_{c,j} = a \sum_{j=1}^i p^j + b \sum_{j=1}^i q^j + \sum_{j=1}^i c \\ &= \frac{ap}{p-1} p^i + \frac{bq}{q-1} q^i + ci + \frac{ap}{1-p} + \frac{bq}{1-q} \end{aligned} \quad (35b)$$

and

$$YY_{0,i} = \sum_{j=1}^i Y_{0,j} \quad (36a)$$

$$\begin{aligned} YY_{c,i} &= \sum_{j=1}^i Y_{c,j} = a \left(\frac{p}{1-p} \right)^2 p^i + b \left(\frac{q}{1-q} \right)^2 q^i \\ &\quad + \frac{1}{2} ci^2 + \left(\frac{ap}{1-p} + \frac{bq}{1-q} + \frac{1}{2} c \right) i \\ &\quad - a \left(\frac{p}{1-p} \right)^2 - b \left(\frac{q}{1-q} \right)^2. \end{aligned} \quad (36b)$$

p and q can be found by finding the values of α_k ($k = 1 \dots 5$) which best fit the n sets of linear equations with respect to α_k :

$$\begin{aligned} \alpha_1 Y_{0,i} + \alpha_2 YY_{0,i} + \alpha_3 i^2 + \alpha_4 i + \alpha_5 &= y_{0,i} \\ (i &= 1, 2, 3 \dots n). \end{aligned} \quad (37a)$$

If one substitutes $YY_{c,i}$ for $YY_{0,i}$, $Y_{c,i}$ for $Y_{0,i}$, and $y_{c,i}$ for $y_{0,i}$, equation (37a) assumes the form

$$\begin{aligned} \beta_1 ap^i + \beta_2 bq^i + \beta_3 i^2 + \beta_4 i + \beta_5 &= 0 \\ \beta_1 &= 1 + \alpha_1 \frac{p}{1-p} - \alpha_2 (p/(1-p))^2 \\ \beta_2 &= 1 + \alpha_1 \frac{q}{1-q} - \alpha_2 (q/(1-q))^2 \\ \beta_3 &= -\frac{1}{2} c \alpha_2 - \alpha_3 \\ \beta_4 &= -\alpha_1 c - \alpha_2 \left(\frac{ap}{1-p} + \frac{bq}{1-q} + \frac{1}{2} c \right) - \alpha_4 \\ \beta_5 &= c - \alpha_1 \left(\frac{ap}{1-p} + \frac{bq}{1-q} \right) \\ &\quad - \alpha_2 \left[a \left(\frac{p}{1-p} \right)^2 + b \left(\frac{q}{1-q} \right)^2 \right] - \alpha_5. \end{aligned} \quad (37b)$$

Equation (37b) can be satisfied for all n values of i if and only if all $\beta_1 = \beta_2 = \beta_3 = \beta_4 = \beta_5 = 0$. Setting β_1 and β_2 to zero yields the characteristic second order polynomial equation, which is equivalent for p and q . The initial estimates for p and q are found according to:

$$p, q = \frac{2 - \alpha_1 \pm \sqrt{\alpha_1^2 + 4\alpha_2}}{2 - 2\alpha_1 - 2\alpha_2}. \quad (38)$$

The values of α_k ($k = 1 \dots 5$) best describing equation for all n values of i are found by minimizing the sum of squares SSQ of equation (37a) for α_k ($k = 1 \dots 5$).

$$\text{SSQ} = \sum_{i=1}^n (y_{0,i} - \alpha_1 Y_{0,i} - \alpha_2 Y Y_{0,i} - \alpha_3 i^2 - \alpha_4 i - \alpha_5)^2$$

$$= \text{minimal.} \quad (39)$$

The solution of this linear least squares problem is given by equation (40) at the bottom of the page.

Although this initial estimation procedure is very robust, there are situations that yield extreme initial estimates (i.e. $p, q < 0.5$ or $p, q > 0.99$). In these situations, the analysis is started using $p = 0.9$ and $q = 0.7$ as starting values. Even in these cases the minimization leads to a good fit at the cost of more iterations. A comparison of the initial Prony-estimates and the final values of p and q , will be discussed in section 5.2.

2.2.2. Error analysis. The quality of the fit is estimated by the Res and Corr parameters as given in equations (15) and (16) with substitution of equation (42) (see below) for SSQ(min).

The error analysis is completely analogous to that described in section 2.1.2 yielding the variance/covariance matrix **MM** (see equation (41) at the bottom of the page).

No new summations need to be calculated for the error analysis since they are available from the preceding fit pro-

cedure, thus yielding a fast error analysis. The error of any function of the five parameters $\bar{a}, \bar{p}, \bar{b}, \bar{q}$ and $\bar{c}$ ($f(\bar{a}, \bar{p}, \bar{b}, \bar{q}, \bar{c})$) may be estimated according to

$$s_f^2 = \sum_{k=1}^5 \sum_{\ell=1}^5 mm_{k,\ell} \left(\frac{\partial f}{\partial \theta_k} \right)_{\bar{\theta}} \left(\frac{\partial f}{\partial \theta_\ell} \right)_{\bar{\theta}} \quad \bar{\theta} = \begin{pmatrix} \bar{a} \\ \bar{p} \\ \bar{b} \\ \bar{q} \\ \bar{c} \end{pmatrix}. \quad (43)$$

In this formula $mm_{k,\ell}$ are the elements of matrix **MM**. From this equation the estimated errors in α , τ_1 , β , τ_2 and c are given by equations (44–49), respectively:

$$s(\alpha) = \left[\left(\frac{\partial \alpha}{\partial \bar{a}} \right)^2 mm_{11} + 2 \left(\frac{\partial \alpha}{\partial \bar{a}} \right) \left(\frac{\partial \alpha}{\partial \bar{p}} \right) mm_{12} + \left(\frac{\partial \alpha}{\partial \bar{p}} \right)^2 mm_{22} \right]^{1/2} \quad (44)$$

with

$$\left(\frac{\partial \alpha}{\partial \bar{p}} \right) = \sigma \bar{a} \frac{(1 + \ln \bar{p})(\bar{p}^\sigma - 1) - \sigma \bar{p}^\sigma \ln \bar{p}}{(\bar{p}^\sigma - 1)^2}$$

$$\left(\frac{\partial \alpha}{\partial \bar{a}} \right) = \frac{\bar{p} \ln \bar{p}^\sigma}{\bar{p}^\sigma - 1} \quad (45)$$

and

$$s(\tau_1) = \left| \left(\frac{\partial \tau_1}{\partial \bar{p}} \right) \right| \sqrt{mm_{22}} = \frac{\Delta t}{\bar{p} \ln^2 \bar{p}} \sqrt{mm_{22}} \quad (46)$$

$$\begin{pmatrix} \alpha_1 \\ \alpha_2 \\ \alpha_3 \\ \alpha_4 \\ \alpha_5 \end{pmatrix} = \begin{bmatrix} \sum_{i=1}^n Y_{0,i}^2 & \sum_{i=1}^n Y Y_{0,i} Y_{0,i} & \sum_{i=1}^n i^2 Y_{0,i} & \sum_{i=1}^n i Y_{0,i} & \sum_{i=1}^n Y_{0,i} \\ \sum_{i=1}^n Y Y_{0,i} Y_{0,i} & \sum_{i=1}^n Y Y_{0,i}^2 & \sum_{i=1}^n i^2 Y Y_{0,i} & \sum_{i=1}^n i Y Y_{0,i} & \sum_{i=1}^n Y Y_{0,i} \\ \sum_{i=1}^n i^2 Y_{0,i} & \sum_{i=1}^n i^2 Y Y_{0,i} & \sum_{i=1}^n i^4 & \sum_{i=1}^n i^3 & \sum_{i=1}^n i^2 \\ \sum_{i=1}^n i Y_{0,i} & \sum_{i=1}^n i Y Y_{0,i} & \sum_{i=1}^n i^3 & \sum_{i=1}^n i^2 & \sum_{i=1}^n i \\ \sum_{i=1}^n Y_{0,i} & \sum_{i=1}^n i Y Y_{0,i} & \sum_{i=1}^n i^2 & \sum_{i=1}^n i & n \end{bmatrix}^{-1} \begin{pmatrix} \sum_{i=1}^n Y_{0,i} y_{0,i} \\ \sum_{i=1}^n Y Y_{0,i} y_{0,i} \\ \sum_{i=1}^n i^2 y_{0,i} \\ \sum_{i=1}^n i y_{0,i} \\ \sum_{i=1}^n y_{0,i} \end{pmatrix}. \quad (40)$$

$$\mathbf{MM} = \frac{\text{SSQ}(\min)}{n-5} \begin{bmatrix} \sum_{i=1}^n \bar{p}^{2i} & \frac{\bar{a}}{\bar{p}} \sum_{i=1}^n i \bar{p}^{2i} & \sum_{i=1}^n (\bar{p} \bar{q})^i & \frac{\bar{b}}{\bar{q}} \sum_{i=1}^n i (\bar{p} \bar{q})^i & \sum_{i=1}^n \bar{p}^i \\ \frac{\bar{a}}{\bar{p}} \sum_{i=1}^n i \bar{p}^{2i} & \left(\frac{\bar{a}}{\bar{p}} \right)^2 \sum_{i=1}^n i^2 \bar{p}^{2i} & \frac{\bar{a}}{\bar{p}} \sum_{i=1}^n i (\bar{p} \bar{q})^i & \frac{\bar{a} \bar{b}}{\bar{p} \bar{q}} \sum_{i=1}^n i^2 (\bar{p} \bar{q})^i & \frac{\bar{a}}{\bar{p}} \sum_{i=1}^n i \bar{p}^i \\ \sum_{i=1}^n (\bar{p} \bar{q})^i & \frac{\bar{a}}{\bar{p}} \sum_{i=1}^n i (\bar{p} \bar{q})^i & \sum_{i=1}^n \bar{q}^{2i} & \frac{\bar{b}}{\bar{q}} \sum_{i=1}^n i \bar{q}^{2i} & \sum_{i=1}^n \bar{q}^i \\ \frac{\bar{b}}{\bar{q}} \sum_{i=1}^n i (\bar{p} \bar{q})^i & \frac{\bar{a} \bar{b}}{\bar{p} \bar{q}} \sum_{i=1}^n i^2 (\bar{p} \bar{q})^i & \frac{\bar{b}}{\bar{q}} \sum_{i=1}^n i \bar{q}^{2i} & \left(\frac{\bar{b}}{\bar{q}} \right)^2 \sum_{i=1}^n i^2 \bar{q}^{2i} & \frac{\bar{b}}{\bar{q}} \sum_{i=1}^n \bar{q}^i \\ \sum_{i=1}^n \bar{p}^i & \frac{\bar{a}}{\bar{p}} \sum_{i=1}^n i \bar{p}^i & \sum_{i=1}^n \bar{q}^i & \frac{\bar{b}}{\bar{q}} \sum_{i=1}^n \bar{q}^i & n \end{bmatrix}^{-1} \quad (41)$$

with

$$\text{SSQ}(\min) = \sum_{i=1}^n \{ \bar{a} \bar{p}^i + \bar{b} \bar{q}^i + \bar{c} - y_{0,i} \}^2. \quad (42)$$

Figure 1. Structure of the decay programs.

$$s(\beta) = \left[\left(\frac{\partial \beta}{\partial \bar{b}} \right)^2 mm_{33} + 2 \left(\frac{\partial \beta}{\partial \bar{b}} \right) \left(\frac{\partial \beta}{\partial \bar{q}} \right) mm_{34} + \left(\frac{\partial \beta}{\partial \bar{q}} \right)^2 mm_{44} \right]^{1/2} \quad (47a)$$

with

$$\left(\frac{\partial \beta}{\partial \bar{q}} \right) = \sigma \bar{b} \frac{(1 + \ln \bar{q})(\bar{q}^\sigma - 1) - \sigma \bar{q}^\sigma \ln \bar{q}}{(\bar{q}^\sigma - 1)^2}$$

and

$$\left(\frac{\partial \beta}{\partial \bar{b}} \right) = \frac{\bar{q} \ln \bar{q}^\sigma}{\bar{q}^\sigma - 1} \quad (47b)$$

$$s(\tau_2) = \left| \left(\frac{\partial \tau_2}{\partial \bar{q}} \right) \right| \sqrt{mm_{44}} = \frac{\Delta t}{\bar{q} \ln^2 \bar{q}} \sqrt{mm_{44}} \quad (48)$$

$$s(c) = |mm_{55}|. \quad (49)$$

The fractional contributions of the first decaying component (f_1) and of the second decaying component (f_2) are calculated from equation (50) and their estimated standard errors are given in equation (51):

$$f_1 = \frac{\alpha}{\alpha + \beta} \quad f_2 = 1 - f_1 = \frac{\beta}{\alpha + \beta} \quad (50)$$

$$s(f_1) = s(f_2) = \frac{1}{(\alpha + \beta)^2} \left\{ \beta^2 \left(\frac{\partial \alpha}{\partial \bar{a}} \right)^2 mm_{11} \right.$$

```

(A) /usr/people/person/data/010896.5
51      # of subimages (dummy if available in file header)
4      left pixel kill
4      right pixel kill
(B) 4      top pixel kill
4      bottom pixel kill
0      smooth(0=no,10=semi-uniform,11=uniform,1-9=perc.)
(C) 2      first subimage to analyse
31     last subimage to analyse
(D) 1      subimage used as blank (if < 0, then -blankvalue)
2      subimage for tests
(E) 300     low intensity threshold
3000    high intensity threshold
7.5     period of images
7.5     integration time
0      save data as image [yes=1, no=0]
/usr/people/person/data/010896.5out
2      output subimage range: start
31     end
(F) 2      skip
0      invert output subimages [yes=1, no=0]
20     low thresholdvalue decay times
40     high thresholdvalue decay times
2      fit with 1 or 2 components [1, 2]
0      if 2 comp., is the 2nd tau fixed [yes=1, no=0]? (else dummy)
15     value for fixed 2nd tau (else dummy)
_____/usr/people/person/data/DECAY.log

```

Figure 2. ASCII input file for decay programs.

$$\begin{aligned}
& +\beta^2 \left(\frac{\partial \alpha}{\partial \bar{p}} \right)^2 mm_{22} + \alpha^2 \left(\frac{\partial \beta}{\partial \bar{b}} \right)^2 mm_{33} \\
& +\alpha^2 \left(\frac{\partial \beta}{\partial \bar{q}} \right)^2 mm_{44} + 2 \left[\beta^2 \left(\frac{\partial \alpha}{\partial \bar{a}} \right) \left(\frac{\partial \alpha}{\partial \bar{p}} \right) mm_{12} \right. \\
& -\alpha \beta \left(\frac{\partial \alpha}{\partial \bar{a}} \right) \left(\frac{\partial \beta}{\partial \bar{b}} \right) mm_{13} - \alpha \beta \left(\frac{\partial \alpha}{\partial \bar{a}} \right) \left(\frac{\partial \beta}{\partial \bar{q}} \right) mm_{14} \\
& -\alpha \beta \left(\frac{\partial \alpha}{\partial \bar{p}} \right) \left(\frac{\partial \beta}{\partial \bar{b}} \right) mm_{23} - \alpha \beta \left(\frac{\partial \alpha}{\partial \bar{p}} \right) \left(\frac{\partial \beta}{\partial \bar{q}} \right) mm_{24} \\
& \left. + \beta^2 \left(\frac{\partial \beta}{\partial \bar{b}} \right) \left(\frac{\partial \beta}{\partial \bar{q}} \right) mm_{34} \right]^{1/2}. \quad (51)
\end{aligned}$$

3. Structure of the DECAY(2L) program

The structure of the DECAY(2L) analysis program is depicted in figure 1. In addition to an image data file several inputs are required. These are organized into an ASCII-text file which can be edited to ensure accurate analysis (see figure 2). The program starts by asking the data filename and the number of subimages or data points (equals n in equations in section 2) (input A, figure 2). Some software programs that are provided to control CCD cameras allow this number to be set in the header of the data file and in these cases the second input will be ‘overruled’ with the contents of the data file header. After this input, the *Datafilter* subroutine is called; it is able to decipher four types of data files and calls the appropriate reading subroutines to read the data files.

Figure 3. Image output of decay programs.

The program proceeds with an optional suppression (‘killing’) of a selectable number of data columns or rows at the top, bottom, left or right positions of the data subimages and an optional smoothing of the data (input B, figure 2).

The killing and smoothing operations are implemented in the *kill_smooth* subroutine and only affect the data analysis and not the original data. The smoothing is done by several 3×3 pixel size filtering operations as a linear 3×3 uniform filtering (all elements $\frac{1}{9}$), a semi-uniform filtering (central element $\frac{3}{15}$, direct neighbors $\frac{2}{15}$, corner or diagonal elements $\frac{1}{15}$), or a non-linear (edge preserving) percentile filtering.

Input C prompts for the subimage range to be analysed (first and last subimage) and the blank subroutine asks for a blank subimage number (input D). If no blank subimage is incorporated in the data image sequence, a value can be given for the camera bias which is then subtracted from the data prior to analysis.

The subroutine *testimage* prompts for a subimage number to select the intensity thresholds for data analysis. Data (or pixels) in this subimage with a higher intensity than the upper threshold or a lower intensity than the lower threshold are not analysed and are represented with a pixel value of -1 in the output (input E).

Prior to starting the analysis pixel loops the program commences with input-block *F*. It requires Δt (or period of images) and δt (integration time), an output filename, and several parameters that influence the output image. These parameters are the first and last subimage for the output, the number of source subimages to skip between two output images, an optional invert of the output images (inverts x and y dimension), and thresholds for the decay time constants. If decay time constants are calculated outside these threshold values, they are not displayed in the output image (pixel value -3). The last inputs determine whether a single or double exponential fit will be performed. In the case of a double exponential, one of the time constants can be optionally fixed and set by the user. The minimization and error analysis in this case is different than described for the two-component fit and is not shown in this paper. The input file concludes with the name of the ASCII log file in which all the average fit parameters are summarized.

Depending on the number of components to be fitted (input F) the program calls the *loopdec* subroutine (for single exponential fit) or the *loopdec2l* subroutine (for double exponential fit). Both subroutines are pixel-by-pixel loops for fitting the data and performing error analysis (*calctau* or *calctau2l*, see section 2 for a description), calculating the output intensity subimages (*filldecayspix* or *filldecayspix2l*) and calculating the output results-of-fit-subimages (*fillirdecay* or *fillirdecay2l*). The output image structure is given in figure 3. In the *fillirdecay* and *fillirdecay2l* subroutines also summations are calculated in order to calculate the average image statistics of a parameter u such as $\sum_m u$ and $\sum_m u^2$ (m represents the number of analysed pixels). After completion of the *loopdec* or *loopdec2l* subroutine, the average ($\langle u \rangle = \sum_m u/m$), standard deviations (SD, see equation (52)), coefficients of variation (CV, see equation (53)) and the true standard deviation (SD_{true} see equation (54)) of a parameter u can

be calculated from these summations; $s(u)$ in equation (54) represents the estimated standard error in parameter u (see equations (23–25) and (44–51)):

$$SD \langle u \rangle = \sqrt{\frac{\sum_m u^2 - m \langle u \rangle^2}{m - 1}} \quad (52)$$

$$CV \langle u \rangle = \frac{SD \langle u \rangle}{\langle u \rangle} \times 100\% \quad (53)$$

$$SD_{\text{true}} \langle u \rangle = \left[(SD \langle u \rangle)^2 - \frac{1}{n - r} \langle (s(u))^2 \rangle \right]^{1/2} \quad (54)$$

$r = 3 \text{ (decay)} \quad r = 5 \text{ (decay2l)}.$

These average image parameters are printed on the screen and put in the ASCII log file by the *outdecay* subroutine. And, if selected, the *writeout* subroutine writes the output image file with all the results (see figure 3) preceded by a header of 512 bytes that contains all the average parameters (see table 1).

4. Image processing

In earlier papers we described image processing using the program TCL-Image (TPD; Technical University of Delft, Delft, The Netherlands) implemented on a DEC MicroVax II computer [21,33]. Hence, the DECAY analysis and image processing were carried out by separate programs. The inconvenience of such a structure was that long output files (see figure 3) had to be generated by the analysis program, and these in turn had to be read and processed by the TCL-Image routines.

In the current situation, the analysis routine has been attached to the commercial image processing package SCIL-Image (TPD, Delft, The Netherlands) which is implemented on powerful Silicon Graphics Inc. (SGI) workstations. The original Fortran source code has been translated into the C language by the f2c tool. The image processing routines that were implemented in TCL-Image had to be rewritten completely in C and are now fully integrated with the analysis routines in the SCIL-Image environment. Minor changes to the Fortran source code were necessary prior to the f2c translation procedure, especially for the file input–output routines that were different for the new computer (SGI) environment. The processing routines work on a 3-D-result image (see figure 3) placed in the computer memory by the analysis routines. In this way the writing and reading of the large output file can be skipped, and one can employ the user-friendly command interfaces provided by SCIL-Image. It should be emphasized, however, that the DECAY(2L) programs, as described in sections 2 and 3, can be used as stand-alone software and hence are not dependent on any image-processing environment.

The image processing routines, currently implemented in SCIL-Image (and earlier in TCL-Image), convert the 3-D result image into a colored 2-D image. Text is

Table 1. Description of output file header (total length 512 bytes). $\tau(s) = (\text{value of pixel}) * (\text{Header}(43) - \text{Header}(42)) / \text{Header}(57) + \text{Header}(42)$, $f_1(\%) = (\text{value of pixel}) * 100 / \text{Header}(57)$, $s_{f_1}(\%) = (\text{value of pixel}) * 100 / \text{Header}(57)$.

Byte	Header	Description
3	2	Width of output image (short)
5	3	Height of output image = header(33) $\times$ height of subimage (short)
65	33	No. of subimages ($N_{\text{tot}} = 4 \times N + 4$ (DECAY) or $4 \times N + 12$ (DECAY2L)) (short)
69	35	Lowest intensity in blank subimage (short)
71	36	Highest intensity in blank subimage (short)
73	37	Lowest intensity in test subimage (short)
75	38	Highest intensity in test subimage (short)
77	39	Lower intensity threshold (short)
79	40	Upper intensity threshold (short)
81	41	Highest intensity in output image (short)
83	42	Lower τ threshold (short)
85	43	Upper τ threshold (short)
87	44	Header(33) (short)
89	45	Subimage width (= header(2)) (short)
91	46	Subimage height (= header(3)/header(33)) (short)
93	47	N (number of intensity images) (short)
95	48	Number of subimages skipped in output image (short)
97	49	First intensity subimage in output image (short)
99	50	Last intensity subimage in output image (short)
101	51	Number of decay times (DECAY = 1, DECAY2L = 2) (short)
109	55	Number of analysed subimages (short)
111	56	Number of blank image (short)
113	57	Scaling factor for result images (short)
117	59,60	Average τ (DECAY) or τ_1 (DECAY2L) (s, float)
121	61,62	Coefficient of deviation of τ (DECAY) or τ_1 (DECAY2L) (% , float)
125	63,64	Average standard error of τ (DECAY) or τ_1 (DECAY2L) (s, float)
129	65,66	Average variance of τ (DECAY) or τ_1 (DECAY2L) (s^2 , float)
133	67,68	True standard deviation of τ (DECAY) or τ_1 (DECAY2L) (s, float)
137	69,70	Minimal calculated τ (DECAY) or τ_1 (DECAY2L) (s, float)
141	71,72	Maximal calculated τ (DECAY) or τ_1 (DECAY2L) (s, float)
145	73,74	0 (DECAY) or average f_1 (DECAY2L) (% , float)
149	75,76	0 (DECAY) or coefficient of variation of f_1 (DECAY2L) (% , float)
153	77,78	0 (DECAY) or average standard error of f_1 (DECAY2L) (% , float)
157	79,80	0 (DECAY) variance of f_1 (DECAY2L) ($\%^2$, float)
161	81,82	0 (DECAY) true standard deviation of f_1 (DECAY2L) (% , float)
165	83,84	0 (DECAY) minimal f_1 (% , float)
169	85,86	0 (DECAY) maximal f_1 (DECAY2L) (% , float)
173	87,88	Average correlation coefficient (float)
177	89,90	Average residual (% , float)
181	91,92	0 (DECAY) or average τ_2 (DECAY2L) (s, float)
185	93,94	0 (DECAY) or coefficient of deviation of τ_2 (DECAY2L) (% , float)
189	95,96	0 (DECAY) or minimum τ_2 (DECAY2L) (ns, float)
193	97,98	Integration time (s, float)
197	99,100	Total number of pixels in one subimage (= header(45) $\times$ header(46)) (float)
201	101,102	Number of analysed pixels (float)
205	103,104	Average number of iterations for initial estimate (float)
209	105,106	Average number of iterations for non-linear fit (float)
213	107,108	Time between subimages (s, float)
217	109,110	0 (DECAY) or maximal calculated τ_2 (DECAY2L) (s, float)
221	111,112	Number of pixels where blank intensity was higher than data (long)
225	113,114	Number of thresholded pixels (long)
229	115,116	Number of pixels with error in initial estimate (long)
233	117,118	Number of pixels with error in τ non-linear fit (long)
237	119,120	Number of pixels with error in global statistics (long)
241	121,122	Number of pixels with too high τ standard error (long)
245	123,124	0 (DECAY) or average standard error of τ_2 (DECAY2L) (s, float)
249	125,126	0 (DECAY) or average variance of τ_2 (DECAY2L) (s^2 , float)
253	127,128	0 (DECAY) or true standard deviation of τ_2 (DECAY2L) (s, float)

added to identify the different result images. Histograms are made of fluorescence intensities, the decay time constants and, in the case of a two component analysis, of fractional contributions. Also two-dimensional histograms are generated depicting correlation between the fitted parameters in the images. By means of the SCIL-Image command interface, the user is able to select several modes of output to suit his need. The final processed images can be exported as TIFF files which can be easily incorporated in several software packages on PCs or Macintosh computers for further processing or incorporation into text documents.

5. Results and discussion

5.1. Computation times

The programs were implemented on an IndyTM Studio workstation (Silicon Graphics, Inc., Mountain View, CA, USA) containing a MIPS R5000/180 MHz processor, using the Irix 6.2 operating system. The speed of the stand-alone Fortran programs was determined by using the profile option ($-p$) in the f77 compilation procedure. The execution time is proportional to the number of analysed pixels. For the single exponential decay (DECAY) program, the average execution time per pixel was 0.129 ms for 50 time points ($n = 50$), including data input and writing of the log file, and excluding writing of the output file (*writeout*). A similar timing for the double exponential program (DECAY2L) yielded a computation time of 1.37 ms/pixel for $n = 100$. The stand-alone C translated programs were also timed and yielded execution times per pixel of 166 μ s and 1.12 ms for the DECAY and DECAY2L programs, respectively. Surprisingly, the translated C code was faster than the original Fortran code for the DECAY2L program, whereas the C code was slower for the DECAY program. The reason for these differences in speed is not known. Hence, for the 100×100 pixel simulations as shown in figures 4 and 5, the total calculation times were 1.7 and 11.2 s respectively, excluding image processing. The image processing was not timed as it is not very dependent on the sizes of the images and usually takes about 1–2 s. Because of the short calculation times we seldom write output images as they are so easy to reconstruct from the original data.

The frequency domain fluorescence lifetime imaging analysis program SINUS(2L) as described in an earlier paper [25], has been similarly incorporated into SCIL-Image, partly making use of identical routines as described here (e.g. for reading microscope data, applying thresholds etc), see also [51]. As mentioned in the discussion of [25], the speed of analysis has been improved over 30-fold, yielding a execution time of 70 μ s/pixel for the single component fit and 87 μ s/pixel for the double-component fit.

5.2. Data simulation

For both single and double exponential cases, simulated data were generated in order to verify the correctness of the fit programs. The noise of the detection device was simulated using $RN = 0.2215\sqrt{I}$ (I is the pixel intensity in an unbinned image), which follows directly from the A/D conversion rate of 1 count per 20.38 electrons of the CCD-camera used. The RN errors were calculated assuming a Poissonian distribution (counting statistics). Figure 4(a) shows the result of the single exponential case. This is a standard image that can be generated from the output data(file) as described in figure 3 after image processing using the *dis.int.decay* image processing command in SCIL-Image. In the simulated data, α was varied from 450 at the top to 8450 counts at the bottom of the image in 10 steps, and the decay time constant τ was varied from 10 s at the left side to 50 s at the right side of the image also in 10 steps. The other parameters were $c = 1/4(\alpha - 250)$, $n = 50$, $\Delta t = 3$ s, $\delta t = 0$ s (equations (1) and (2)). The fit quality is very good as there are no obvious deviations between the simulated data (top row of images) and the reconstructed or calculated data (third row of images) in figure 4(a). This also can be inferred from the absence of intensity in the eight-fold multiplied difference data. From figure 4(b) (also a standard image generated from the output data(file) as described in figure 3 after image processing using the *DECAY.analysis* image command) it is inferred that the DECAY program indeed accurately determines α , c and τ . Each unique combination of α and τ is identified, as can be seen from the two-dimensional histogram showing the correlation between α and τ . From the temporal histograms and the two-dimensional histograms it is clear that the camera noise does not contribute significantly to a spread in the calculated parameters. The average image statistics (see table 2) supports this statement as the average standard error in all determined time constants is only 0.11 s. The calculated coefficient of variation and standard deviation in τ are exactly the same as the expected values of 42.46% and 12.76 s, respectively. Note that on average 0.27 iterations are used in the minimization procedure as described in section 2.1, which means that four out of five times the initial estimate procedure (section 2.1.1) yields the optimal fit. The average difference of the initial estimate and the final value of τ was quantified and amounted to only 0.07%. Hence, it may be concluded that no further minimization is necessary after the initial estimate. We, however, did not omit the minimization procedure in the program since the summations are calculated that are used for the error analysis.

The results of the two-component simulation analysis are presented in figure 5. In the simulated data α was varied from 1000 at the top to 10000 at the bottom of the image whereas β was varied in the opposite direction; τ_1 was set at 10 s and τ_2 was varied from 20 s at the left to

(a)

(b)

Figure 4. Analysis of simulated data with single component decay. (a) Intensity subimages; (b) Result images.

100 s at the right side of the image. Further parameters were $c = 1/5\alpha$, $n = 100$, $\Delta t = 1.5$ s, $\delta t = 0$ s. Figure 5(a) shows the comparison between the simulated data and the reconstructed data. Also here no apparent deviations can be seen. The calculated values of α , β , τ_1 , τ_2 , and c are depicted in figure 5(b). By comparing figures 5(a) and 5(b) it is clear that for all combinations of α , β , τ_1 , τ_2 , and c the simulated data and the parameters are reconstructed accurately. Only for combinations with a

very low f_1 and a ratio of $\tau_2/\tau_1^2 \leq 2$ in some cases the fit gave erroneous results represented by cyan colored pixels in the output images. The spread in the time constants caused by the RN noise is acceptable, as can be seen from the histograms in figure 5(b) and the average image statistics in table 2. The calculated coefficient of variation and true standard deviation of τ_2 are very close to the expected values of 42.56% and 25.3 s, respectively. From inspection of the two-dimensional histogram depicting the

(a)

(b)

Figure 5. Analysis of simulated data with two-component decay. (a) Intensity subimages. (b) Result images. (c) Comparison of initial estimates and final values of τ_1 and τ_2 .

correlation between β and τ_2 , it can be inferred that at a ratio of $\tau_2/\tau_1 = 2$ the β becomes less well defined. The average fit parameters (see table 2) show that on average only nine iterations are used after the initial estimate to solve equation (30). In figure 5(c), the initial estimates

of τ_1 and τ_2 are compared with the final values of τ_1 and τ_2 . The cyan error pixels in the left pair of images indicate that the Prony-like estimation procedure yielded erroneous results ($p, q < 0.5$ or $p, q > 0.99$). In these pixels, the fit procedure was initiated using $p = 0.9$ and

(c)

Figure 5. Continued.

$q = 0.7$. The subsequent fitting procedure found a correct value for τ_1 and τ_2 (compare the left and middle pair of images in figure 5(c)) for almost all of these ‘difficult’ pixels demonstrating its robustness. The decrease in noise in the middle pair of the τ_1 and τ_2 images as compared to the left ones of figure 5(c), clearly indicates that the minimization procedure provides a further optimization of the time constants. The relative changes in τ_1 and τ_2 are depicted in the right pair of images. The average changes were 13.2% and 10.4%, respectively. From the right pair of images, it is evident that the Prony-like method of parameter estimation becomes less reliable in the case of $\tau_1 \simeq \tau_2$ or when f_1 is low. In these cases the observed decay curve (with evenly spaced data) is close to a single exponential decay. We want to stress, however, that for almost all of the other combinations of the parameters, the initial estimates are quite good and thereby this unbiased (requiring no start values from the user) procedure greatly contributes to the speed of the two-component fit algorithm.

5.3. Experimental data

Several papers have appeared using the single exponential analysis program DECAY as described above [21, 24, 29, 31–35]. The applicability of these programs for

TRIM has been discussed in those papers. In this section we further illustrate the use of the DECAY program for the sensitive detection of FRET by photobleaching microscopy. The basis of FRET is the transfer of excited state energy from a donor fluorophore to an appropriate acceptor molecule, and only occurs over very short distances (generally < 10 nm). FRET is manifested in many different ways: a decreased donor quantum yield, a decreased fluorescence lifetime, and an increased stability towards chemical photobleaching [24, 29, 34, 35, 52–55]. The increased stability towards photobleaching can be quantified by measuring the increase in decay time constants describing the photobleaching process. We applied this technique to study the dimerization of the epidermal growth factor receptor (EGFR) on living human epidermoid carcinoma A431 cells. The cell surface EGFRs were labeled with fluorescein-EGF (Fl-EGF, donor) in the presence and absence of rhodamine-EGF (Rh-EGF, acceptor). Receptor dimerization is accompanied by an increase in photobleaching time constants. An extensive study has appeared elsewhere [21]. In figure 6(a) the progress of photobleaching is shown for both the donor in the absence (left side of the subimages) and the presence of acceptor (right side of the subimages) in a (digitally) merged experiment. From figure 6(a) it is apparent that even for the complex cellular

(a)

(b)

Figure 6. Detection of FRET by photobleaching microscopy of Fl-EGF bound to A431 cells in the presence of a two-fold excess of native EGF (left subimages, $-A$) or of Rh-EGF (right subimages, $+A$). (a) Fluorescence intensity subimages; (b) Result images. A431 cells grown on glass coverslips (for culture conditions see [21]), were incubated with a mixture of 16.7 nM Fl-EGF and either 33.3 nM native EGF (left images) or 33.3 nM Rh-EGF (right images) at 4°C for 40 min and then for 6 min at 20°C. The cells were fixed and mounted as described [21]. Two sequences of 31 images (72×116 pixels, after 3×3 binning, corresponding to an area of $34 \times 55 \mu\text{m}$ in the object plane) were acquired for the respective incubations, the first image with excitation off (blank), the others with exposure to excitation light for 7.5 s (Δt) and integration of the first 1.5 s (δt) of the fluorescence emission from Fl-EGF only, on the CCD camera, using the microscope-CCD camera system as described [21]. Prior to analysis and processing, the image sequences were digitally merged into one image sequence. Analysis and processing was similar to that described in figures 4(a) and (b), for average image results see table 2.

environment, the decaying intensities in the images can be described accurately by a single exponential, since there is hardly any deviation of the reconstructed data (third row of images) from the observed data (e.g. second row of images) as shown in the difference images in the fourth row. The green areas in figure 6(a) correspond to areas outside cells that were excluded from the fitting due to thresholding. The results of the fit are shown in figure 6(b). It is immediately apparent from the decay time constant (i.e. τ) image that the photobleaching is retarded in the double labeled cells (right half of the subimages). In the temporal histogram of τ , the two peaks correspond to the respective experiments and are clearly separated in time. In the two-dimensional histogram depicting the correlation between α and τ , it can be seen that pixels with equal fluorescence intensity in the two experiments gave rise to a different τ . The average image parameters from the two separate experiments and from the merged data as shown in figure 6(b) are listed in table 2. These average values indicate that τ increased from 41.0 to 47.5 s in the presence of the acceptor, indicating an average effective FRET efficiency of 14% in the double labeled cells. It is apparent that such low FRET efficiencies are determined easily by photobleaching microscopy (pbDIM) in contrast to (conventional) steady-state measurements of spatially resolved FRET efficiencies [29, 53, 56]. The accuracy attainable with pbDIM is indicated by the average standard error of each individually determined τ (1 s or about 2.5% of the actual values), as well as from the other average image parameters (see table 2). It is noteworthy that in the double labeled cells (right side images), the actual bleaching process is in fact double exponential, since the intensity in each pixel of the image corresponds to a mixture of single and dimerized receptors. Nonetheless, the decay process is described very well with a single exponential due to the low average FRET efficiency. In interpretations of the FRET efficiencies, one has to deal explicitly with this simplification ([21]; see also [20, 23]). From a 14% effective FRET efficiency, it was inferred that under our experimental conditions the extent of EGFR dimerization was 36% [21].

To illustrate the application of the DECAY2L program, a two-component analysis is shown of the photobleaching of Bodipy-Fl-PC in a single living BALB/c-3T3 cell

(figure 7). Due to double labeling with Bodipy-Fl-PC (donor) and an eight-fold excess of Bodipy-530/550-PC (acceptor) FRET should occur in such membrane systems. The nucleus was not stained with Bodipy-Fl-PC (figure 7(a)), as expected. Some pixels (with low intensities) gave rise to errors in the analysis; they are marked with a cyan color. There is hardly any intensity in the difference images between the observed and calculated data (fourth row, figure 7(a)), showing that the fit described the experimental data perfectly. This is also apparent from the average image statistics (see table 2), indicating a residual of about 1.0% and an average correlation of 99.96%, values very close to the ideal. The speed of analysis was the same as for the simulated data as is apparent from the low average number of iterations ($< 10/\text{pixel}$). The calculated parameter images from the fit are shown in figure 7(b). Here one can see that particularly the plasma membrane regions had a much slower decaying second component (τ_2), in contrast to the heavily stained regions surrounding the cell nucleus and corresponding to internal membrane structures (such as endoplasmic reticulum and Golgi apparatus). The program appears to have allocated the slower bleaching kinetics specifically to τ_2 , as is apparent from the rather homogeneous τ_1 image as well as from the symmetrical temporal τ_1 histogram (upper right) and the two-dimensional histograms in figure 7(b) depicting the correlation between τ_1 and α (lower left) or τ_2 (lower right). The increased τ_2 values in the plasma membrane indicate that the absolute concentration (in mol %) of the acceptor dye (Bodipy-530/550-PC) was markedly higher in the plasma membrane than in the internal membranes. Since the distribution of both lipids was similar (data not shown), one can conclude that the surface concentration of both Bodipy-PCs was less in the internal membranes (despite the higher intensities) than in the plasma membrane. This is logical inasmuch the Bodipy-PCs were added exogeneously and therefore stained the plasma membrane first. Thus, the higher intensities in the internal membranes must have reflected the higher amount (mass) of internal membranes, rather than a specific targeting of the lipid to these membranes. The plasma membrane localization of Bodipy-550/550-PC is in marked contrast to that of Bodipy or fluorescein labeled ceramides, which are very efficiently targeted to the Golgi apparatus

Table 2. Average image statistics.

Experiment	τ_1 (s)	CV (%)	$\langle s_{\tau_1,i} \rangle$ (s)	$\langle s_{\tau_1,true} \rangle$ (s)	τ_2 (s)	CV (%)	$\langle s_{\tau_2,i} \rangle$ (s)	$\langle s_{\tau_2,true} \rangle$ (s)	R (%)	res (%)	iter
sim 1 τ	30.00	42.56	0.11	12.76					99.998	0.5	0.27
sim 2 τ	10.00	3.46	0.16	0.34	60.1	42.5	0.92	25.6	100.000	0.3	9.3
EGF-Fl (-A)	41.0	5.2	0.95	2.11					99.86	2.6	1.3
EGF-Fl (+A)	47.5	2.9	1.03	1.36					99.89	2.1	1.0
EGF-FL total	44.8	8.1	1.00	3.64					99.88	2.3	1.1
Bodipy-lipid	13.5	9.9	0.70	1.33	71.8	41.0	9.8	29.3	99.96	1.0	9.8

(a)

Figure 7. Photobleaching of Bodipy-FL-PC labeled living BALB/c-3T3 cells using a two-component analysis. (a) Fluorescence intensity subimages. BALB/c-3T3 fibroblasts were grown in a 5% CO₂ humidified atmosphere at 37 °C in Minimal Essential Medium (MEM with Earls salts, GIBCO, cat. No 21090-022, Gaithersburg, MD, USA) supplemented with 0.1 mg/ml gentamycin, 0.292 mg/ml L-glutamine and 10% (v/v) fetal bovine serum (FBS) on 22 mm ø glass coverslips placed in a petri dish. After washing the cells three times with phosphate buffered saline (PBS), the cells were incubated for 30 min at 37 °C with Hanks Buffered Salts (HBSS, GIBCO) supplemented with 1 mg/ml BSA, 0.1 μ M 1-palmitoyl-2-(4,4-difluoro-5,7,dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-*sn*-glycero-3-phosphocholine (Bodipy-FL-PC, Molecular Probes, Eugene, OR, USA) and 0.8 μ M 1-palmitoyl-2-(4,4-difluoro-5,7,diphenyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-*sn*-glycero-3-phosphocholine (Bodipy-530/550-PC, Molecular Probes, Eugene, OR, USA). The labeling buffer was prepared by injecting an ethanol solution of the Bodipy-PCs mixture into the HBSS buffer (final ethanol concentration was less than 1%). Cells were washed three times with PBS, and mounted in HBSS buffer, and used directly for microscopy. The microscope-CCD camera system used was different from that described in [21]. The system consisted of an HBO 100 W mercury lamp (Osram, Germany) as excitation source; a Leica DMR-RBE fluorescence microscope (Leitz, Wetzlar, Germany) incorporating a Leitz Fluotar 100 $\times$ NA 1.3 oil immersion objective, a D485/15 excitation filter, a 505 nm dichroic mirror, and a D525/20 emission filter (all Chroma, Inc., Brattleboro, VT, USA) for selecting specifically the Bodipy-FL fluorescence; and a slow-scan Series 200 (CH250) CCD camera (Photometrics, Inc., Tucson, AZ) incorporating a thermoelectrically cooled SI 502-AB thinned and back-illuminated CCD sensor with 510 $\times$ 510 (available) square 24 μ m-pixels interfaced to an Apple Macintosh Power PC 7100/66 via the Nu200 controller. Camera control was achieved by the IPLab Spectrum software (Signal Analytics, Vienna, VA, USA). A sequence was taken by acquiring a blank image (excitation off) followed by 60 images of 160 $\times$ 140 pixels with excitation on (no binning), corresponding to an area of 37 $\times$ 32 μ m in the object plane. The time interval between the subimages was 3.56 s (Δt) and the CCD-camera was opened 1 s (δt) for each subimage. Image processing is as described in figure 5. For average results see table 2. (b) Result images; (c) Surface plot of τ_2 .

and accumulate there at surface concentrations above 10 mol %, as has been inferred from excimer formation [57, 58]. The method presented here enables the imaging of *in vivo* lipid concentrations in subcellular structures in the range of 0–1 mol %, and hence is potentially more sensitive than other techniques based on the monitoring of excimer formation [58, 59].

6. Concluding remarks

We have described the development of fast algorithms for decay analysis in a series of digitized images. These general algorithms have been tested thoroughly with simulated image-data featuring many combinations of intensities, time constants and (in the case of a double exponential analysis) of fractional contributions. The simulated data demonstrate

that even without initial conditions, and without any concessions to convergence criteria, the algorithms are capable of handling almost any of the above combinations of parameters with a minimum number of iterative steps and hence execution time. Procedures that solve normal equations directly (e.g. Newton–Raphson) instead of addressing the problem as a non-linear least squares minimization issue (as in the Marquardt method) are known to produce parasitic solutions. However, we have never encountered this situation in our work involving the processing of thousands of image-pixels. The estimation of the decay time constant(s) for each pixel can be considered as an independent realization of a random process to which the estimation procedure is applied (i.e. fits to exponentials). Thus, in the more than 10⁸ applications of our procedure we have not, to our knowledge, found a parasitic solution. We

(b)

(c)

Figure 7. Continued.

conclude that our procedure is robustly protected against such erroneous solutions.

The usefulness of the algorithms for analysing TRIM data was illustrated by two cell biological examples using photobleaching microscopy. From these examples it is clear that by using these algorithms, photobleaching microscopy provides a powerful tool for studying biomolecular interactions in single cells with high sensitivity and spatial resolution. We expect many future applications of this type of TRIM, inasmuch as, in addition to a conventional fluorescence microscope, one requires only a digital camera and modest computer facilities for assembling a complete TRIM system.

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