Improving time domain fluorescence lifetime imaging with an Adaptive Monte Carlo Data Inflation (AMDI) algorithm

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ABSTRACT

Fluorescence Lifetime Imaging Microscopy (FLIM) is a powerful technique which gives access to the local environment of fluorophores in living cells. However, to correctly estimate all lifetime parameters, time domain FLIM imaging requires a high number of photons and consequently a long laser exposure time which is not compatible with the observation of dynamic molecular events and which induces cellular stress phenomena. For reducing this exposure time, we have developed an original approach to statistically inflate the number of collected photon. This approach called Adaptive Monte Carlo Data Inflation (AMDI) combines the well-known bootstrap technique with an adaptive Parzen kernel. We have evaluated its potential on experimental FLIM data *in vivo*. We have demonstrated that our robust method allows estimating precisely fluorescence lifetime with exposure time reduced up to 50 times for mono-exponential (corresponding to a minimum of 20 photons/pixel) and 10 times for bi-exponential decays (corresponding to a minimum of 5000 photons/pixel) in comparison with the standard fitting method. Furthermore, thanks to AMDI, we demonstrate that it becomes possible to estimate accurately all fitting parameters in FRET experiments without constraining any parameter. An additional benefit of our technique is that it improves the spatial resolution of the FLIM images by reducing the commonly used spatial binning factor.

Keywords: Time Correlated Single Photon Counting (TCSPC); Förster Resonance Energy Transfer (FRET); Least Square Method (LSM) curve fitting; Monte Carlo; Parzen kernel; Bootstrap; Fluorescence Lifetime Imaging Microscopy (FLIM); molecular interactions; live cell

1. INTRODUCTION

In addition to intensity and wavelength, lifetime is a supplementary source of contrast in fluorescence microscopy, which gives access to the local environment of fluorophores in living cells. Fluorescence Lifetime Imaging Microscopy (FLIM) has been widely used for quantifying Förster Resonance Energy Transfer (FRET) occurring between interacting proteins in tissues or cells.[1] In order to measure fluorescence lifetime, a large number of different techniques exist which can be classified into two main groups: frequency domain[2] and time domain methods.[3] In this work, we limit our study to this second group.

In the time domain method, the fluorescent sample is excited repeatedly with short pulses of light, and fluorescence decay histograms $I(t)$ are recorded by measuring the time delays between these pulses and the emitted fluorescence photons. Technically, this can be done either by using a time correlated single photon counting (TCSPC) system, or by measuring the fluorescence signal with a specific detector. Whatever technique applied, the theoretical fluorescence intensity profile $I(t)$ is defined by:

$$I(t) = \sum_i a_i \exp\left(-\frac{t}{\tau_i}\right) \quad \text{with} \quad \sum_i a_i = 1 \tag{1}$$

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Medical Laser Applications and Laser-Tissue Interactions V, edited by Ronald Sroka, Lothar D. Lilge, Proc. of SPIE-OSA Biomedical Optics, SPIE Vol. 8092, 80920C
 · doi: 10.1117/12.895331

Where a_i is the contribution and τ_i is the lifetime of the species i. In most cases, the experimental determination of these values is achieved by successively minimizing the difference between the collected data and the theoretical model. When several fluorescent species are present in the sample (as in FRET experiments), the precise estimation of all fitting parameters requires a large number of photons. In order to increase this number of counted photons, two possibilities exist: either increasing the laser power or extending the acquisition time. However both possibilities are harmful; a higher laser power generates photo-damage in living cells and a longer acquisition time involves a longer laser excitation time resulting in cellular stress.

Recently, many efforts have been made to reduce this acquisition time by developing high speed FLIM techniques with specific detectors and dedicated electronic cards.[4–6] In this work, we did not modify the FLIM acquisition system which is a standard TCSPC system. In order to significantly decrease this acquisition time and subsequently improve the FLIM imaging studies in living cells, we propose an original approach based on a statistical data inflation method that we called Adaptive Monte Carlo Data Inflation (AMDI).[7]

2. THE ADAPTIVE MONTE CARLO DATA INFLATION (AMDI) ALGORITHM

Our AMDI approach combines two techniques: the Parzen kernel and the bootstrap technique.

The Parzen kernel is a non-parametric way of estimating the probability density function of a random variable. If $x_1, x_2, ..., x_N$ are independent and identically distributed random variables with a probability density function f then the kernel density approximation of this probability density function is defined by[8]

$$f_g(x) = \frac{1}{N \times g} \sum_{i=1}^{N} k\left(\frac{x - x_i}{g(x)}\right) \tag{2}$$

Where k is a kernel (which a symmetric function whose integral is one) and g is a smoothing function. In this work, we used a standard Gaussian kernel with mean zero and variance 1. Note that the smoothing function g controls indirectly the variance of this Gaussian kernel as

$$k\left(\frac{x - x_i}{g(x)}\right) = \frac{1}{\sqrt{2\pi}} \exp\left(-\frac{(x - x_i)^2}{2g(x)^2}\right) \tag{3}$$

In this study, we used an adaptive smoothing factor over x, which depends on a priori knowledge about the properties of the density function to be estimated. When the total photon number N is low ($N < 5000$ photons), we used the inverse function of the theoretical law of the fluorescence photon emission ($g(x) = \ln(x)$). For higher total photon numbers ($N > 5000$ photons), there are no more empty temporal channels; it is then no more necessary to spread temporally the information as much as previously. We thus used a lower smoothing factor: $g(x) = \log(x)$. In all cases, the width of the Parzen kernel increases when the temporal channel increases in order to maintain an almost constant signal to noise ratio for each temporal channel and to compensate for the poor signal to noise ratio that occurs for longest emission photon time where the photon number is lower.

The bootstrap technique[9] is a statistical inference method. This re-sampling method relies on the generation of a huge number of simulated data samples from an original small experimental data set.

In order to estimate the parameters of the collected fluorescence decays, we combined these two previous techniques in the AMDI algorithm. The AMDI algorithm which generates amplified fluorescence decay histograms from a small data sample is as follows:

The fluorescence decay histogram called $h(t)$ was built from H_m and H_s where H_m represents the measured set of photon arrival events e_i such that $H_m = e_1, e_2, ..., e_p$ (with p, the number of events) and H_s is the simulated set such that $H_s = s_1, s_2, ..., s_q$ (with q, the number of simulated photon events which is at least an order of magnitude greater than p). We call $G(\mu, \sigma)$ a Gaussian random generator with mean μ and standard deviation σ. We assumed that the photon shot noise for a photon event e_i is Poisson distributed and therefore that σ is equal to $g.h(e_i)^{1/2}$ where g is the previously described smoothing function. The AMDI algorithm was the following:

1. Set a loop counter c to 1 and initialize H_s with a size of q.
2. Generate a value i (comprised between 1 and p) from a random number generator with an uniform probability
3. Set x to e_i
4. Set s_c to $G(x, g.h(x)^{1/2})$
5. Add s_c to H_s
6. Increment c
7. While $c < q$ repeat steps 2 to 6
8. Build a new fluorescence decay histogram from new set H_s

This algorithm is applied to all photons constituting the fluorescence intensity decays but not to the photons present in the first temporal channels (which correspond to the background). For these photons, we have calculated the mean number of background counts per pixel, inflated it with the same coefficient factor and spread it in the first temporal channels without smoothing function ($g = 1$).

This AMDI algorithm is implemented into an homemade software named TITAN (IRI, USR 3078 CNRS, BCF).

3. DEMONSTRATION OF THE AMDI BENEFITS FOR FLIM IMAGING IN LIVING CELLS

In order to demonstrate that the AMDI algorithm is efficient for correctly estimating lifetime values *in vivo*, we have performed FLIM images of living cells transfected with gpi-eGFP. The eGFP (N)-terminus was tagged with the mouse Thy-1 glycosylphosphatidylinositol (GPI) anchoring sequence, which directs the fusion protein to the outer leaflet of the plasma membrane (as shown on Fig.1.A and B). In order to reduce the cellular stress, the acquisition time was short (90s) in comparison with standard FLIM experiments (300s), which leads to a small number of collected photons (the mean number of photons per pixel is inferior to 30). For such condition, without AMDI algorithm, the standard fitting method is not able to determine correctly the fluorescence lifetime of the gpi-eGFP when we applied a binning (which consists in summing the intensity profiles of neighbour pixels comprised in the square surface $(2n + 1)^2$) of factor $n = 0$ because the number of photons is too small (image not shown). As shown on Fig. 1.C, when few photons are present per pixel, the mean lifetime of gpi-eGFP is largely overestimated (around 3.6 ns) in comparison with the value $\tau_{mean} = 2.4$ ns obtained in standard FLIM acquisition conditions (300s and $n = 3$).When the AMDI algorithm is applied, the number of photons per pixel is statistically inflated and the accuracy of the mean fluorescence lifetime estimation is thus improved ($\tau_{mean} = 2.4$ ns with $n = 0$) although the standard deviation σ is large ($\sigma = 0.9$ ns).

In order to improve both the lifetime precision and accuracy, it is necessary to increase the number of photons. For this, one can either increase the acquisition time or the spatial binning factor n. As seen in Fig. 1, we have first applied a spatial binning with a factor $n = 1$. When the standard fitting method is employed, the estimation of the mean fluorescence lifetime is improved but still slightly underestimated ($\tau_{mean} = 2.17$ ns and $\sigma = 0.29$ ns). By applying the AMDI algorithm, the mean fluorescence lifetime is still well estimated ($\tau_{mean} = 2.38$ ns) and the standard deviation largely improved ($\sigma = 0.24$ ns). Even if this spatial binning permits to improve the estimation and the precision of the mean lifetime, it is important to notice that the spatial resolution of the FLIM image is degraded (Fig. 1.A1 and 1.B1). With $n = 1$, the lifetime value associated with each pixel corresponds indeed to a surface of 3×3 pixels. Consequently, the FLIM image is subjected to an average filtering and the fluorescence lifetime texture is loss which could create artifacts and misinterpretations.

To avoid this problem and maintain both the best spatial and lifetime resolution possible, we have increased the TCSPC acquisition time to 300s. When the standard fitting method is employed (without binning factor), the low number of photons (whose mean is around 90 photons per pixel) prevents from estimating correctly the

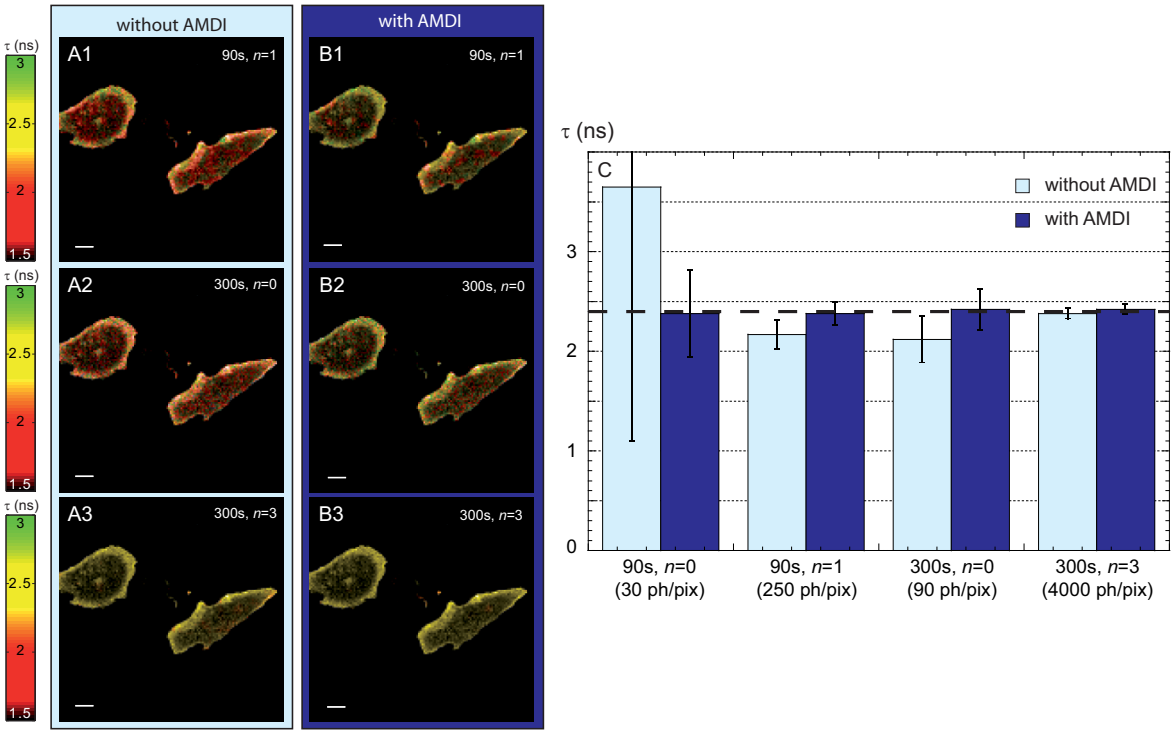

Figure 1. Improvement of fluorescence lifetime measurements *in vivo* with AMDI. We have represented FLIM images of U2OS cells transfected with gpi-eGFP after applying (B) or not (A) the AMDI algorithm. Three distinct conditions were considered: (1) 90s acquisition time with $n = 1$, (2) 300s acquisition time with $n = 0$ and (3) 300s acquisition time with $n = 3$. Scale bar: 10μm. The mean fluorescence lifetime and the standard deviation of each FLIM image are reported in the histograms (C). The mean photon number per pixel is also indicated. This experiment demonstrates that the AMDI algorithm enables to correctly estimate the fluorescence lifetime of gpi-eGFP in living cells even if the number of collected photon is low, which is not possible with the standard fitting method.

fluorescence lifetime. The mean lifetime is thus slightly underestimated ($\tau_{mean} = 2.12$ ns with $\sigma = 0.46$ ns). Thanks to the AMDi algorithm, the correct estimation of the gpi-eGFP fluorescence lifetime ($\tau_{mean} = 2.40$ ns with $\sigma = 0.41$ ns) is possible even if $n = 0$ (Fig.1.C). The spatial resolution of the FLIM image is then preserved and it corresponds to the initial resolution of the FLIM acquisition (Fig.1.B2). In other words, thanks to the AMDI algorithm, we do not need to degrade the spatial resolution of the FLIM image in order to have enough photons for correct lifetime estimation. Analysis artifacts induced by the average spatial filtering become then totally inexistent.

In order to evaluate the potential benefits of the AMDI algorithm for estimating bi-exponential decays *in vivo*, we have finally performed FRET experiments on living HEK293 cells expressing eGFP (donor) linked to mCherry (acceptor) fused to a protein expressed at the membrane (Fig. 2.A and B). The total acquisition time was 300s and we have first applied a spatial binning factor of 5 in order to obtain a mean photon number of approximately 7500 per pixel. This number of photons is not enough to estimate correctly both fluorescence lifetime with the standard fitting procedure (Fig. 2.C). we have therefore measured in a first FLIM experiment the fluorescence lifetime of the donor alone (2.35 ns $\pm$ 0.13 ns) which corresponds to the τ_2 of FRET experiments (data not shown). For estimating the first lifetime value τ_1, we have constrained the second lifetime τ_2 to 2.35 ns and we have performed standard fitting method (data not shown). In this condition, we found $\tau_1 = 1.16$ ns $\pm$ 0.1 ns, which is not in good agreement with the value obtained without AMDI algorithm when all parameters are free ($\tau_1 = 1.38$ ns $\pm$ 0.2 ns and $\tau_2 = 2.8$ ns $\pm$ 0.53 ns). In order to increase the number of photons and to obtain a mean of 20 000 photons per pixel, we applied a binning factor of 10. However, this photon number is not enough for estimating correctly both lifetime values with the standard fitting method. The measured values of $\tau_1 = 1.36$ ns $\pm$ 0.09 and $\tau_2 = 2.87$ ns $\pm$ 0.2 ns are indeed different of the expected ones (cf. Fig.2.C).

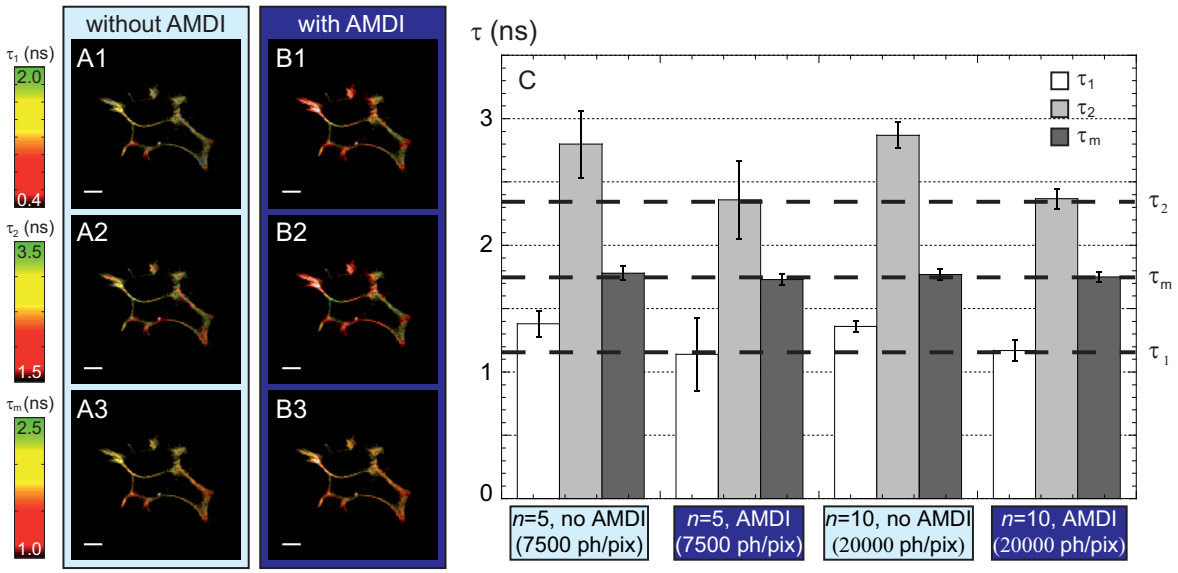

Figure 2. Improvement of FRET measurements *in vivo* with AMDI. We have performed FRET experiments on HEK293 living cells expressing eGFP-mcherry tandem linked to a protein expressed at the membrane. The fluorescence lifetime images estimated without or with AMDI algorithm are represented respectively in (A) and (B). For each case, three FLIM images are shown: (1) the first fluorescence lifetime τ_1, (2) the second fluorescence lifetime τ_2 and (3) the mean lifetime τ_{mean} ($\tau_{mean} = a_1 \times \tau_1 + (1 - a_1) \times \tau_2$). Scale bar: 10$\mu$m and $n = 5$. The median fluorescence lifetime values estimated with $n = 5$ and $n = 10$ are indicated in the histograms (C) and the error bars correspond to the interquartile range . The mean photon number per pixel is also reported. We have also indicated with dashed lines the expected values of the three fluorescence lifetimes: $\tau_1 = 1.16$ ns, $\tau_2 = 2.35$ ns and $\tau_{mean} = 1.75$ ns (see text for details). With the AMDI algorithm, it becomes possible to estimate accurately all fitting parameters without constraining any parameter, which is not possible with the standard fitting method.

When the AMDI algorithm is applied, we are now able to estimate accurately all fitting parameters without constraining any parameter, even if the mean photon number per pixel is around 7500. As indicated in Fig. 2.C, both fluorescence lifetime values ($\tau_1 = 1.14$ ns $\pm$ 0.58 ns and $\tau_2 = 2.36$ ns $\pm$ 0.62 ns) are in good agreement with the values previously described but the interquartile range of these lifetimes is large. We can improve the precision of these measurements by applying a spatial binning factor of 10. With $n = 10$, we obtain: $\tau_1 = 1.17$ ns $\pm$ 0.16 ns and $\tau_2 = 2.37$ ns $\pm$ 0.16 ns. It should be finally noticed that the mean fluorescence lifetime τ_{mean} ($\tau_{mean} = a_1 \times \tau_1 + (1 - a_1) \times \tau_2$) is always well estimated for these number of photons (with or without AMDI algorithm).

In this work, we demonstrated the benefit of the AMDI algorithm by fitting the experimental FLIM data with a Levenberg Marquardt algorithm which represents a good compromise between optimization speed and lifetime precision. However the benefit of the AMDI algorithm is of course not limited to this fitting method. It can indeed be easily extended to all minimization algorithms classically employed in time domain FLIM image analysis (such as the Newton trust region regression method which is more robust to dispersive elements). Moreover, it should be noticed that the AMDI algorithm which is an inflation of temporal fluorescence intensity decays is also compatible with all existing time domain FLIM image analysis strategies.[10,11]

ACKNOWLEDGMENTS

We thank Bernard Vandenbunder (IRI) for fruitful discussions and critical comments on the manuscript. We are grateful to Franck Riquet for providing both gpi-eGFP and memb-eGFP-mCherry plasmids. This work was funded by the CNRS, the Région Nord-Pas de Calais, the European Regional Developmental Funds, the French Research Agency ANR 07-PFTV-01101 and a Leica Microsystems partnership. The Biophotonics team was supported by two national networks: GDR2588 and RT-MFM/MRCT. This work benefited from scientific

discussions during the thematic school MiFoBio. We are grateful to the imaging platform BICFaL (Biophotonic and Imaging Core Facility of Lille).

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