Photobleaching correction in fluorescence microscopy images

Nathalie B Vicente, Javier E Diaz Zamboni, Javier F Adur, Enrique V Paravani and Victor H Casco

Microscopy Laboratory, School of Engineering – Bioengineering, National University of Entre Ríos (UNER). Ruta 11, Km 10 (3101), Oro Verde, Entre Ríos, Argentina.
nathalie.vicente@gmail.com – vcasco@bioingenieria.edu.ar

Abstract. Fluorophores are used to detect molecular expression by highly specific antigen-antibody reactions in fluorescence microscopy techniques. A portion of the fluorophore emits fluorescence when irradiated with electromagnetic waves of particular wavelengths, enabling its detection. Photobleaching irreversibly destroys fluorophores stimulated by radiation within the excitation spectrum, thus eliminating potentially useful information. Since this process may not be completely prevented, techniques have been developed to slow it down or to correct resulting alterations (mainly, the decrease in fluorescent signal). In the present work, the correction by photobleaching curve was studied using E-cadherin (a cell-cell adhesion molecule) expression in Bufo arenarum embryos. Significant improvements were observed when applying this simple, inexpensive and fast technique.

1. Introduction

Even though the terms “fluorophore” and “fluorochrome” are generally used indistinctively, in the present work we will consider a fluorochrome as a fluorescent molecule, while defining a fluorophore as the conjugation of a fluorochrome with a given macromolecule. Fluorophores are fluorescent probes used to detect the expression of proteins and nucleic acids, to which they bind with high specificity. When these molecules are stimulated with electromagnetic waves of particular wavelength (which, as a whole, are known as excitation or absorption spectrum), they re-emit energy at longer wavelengths (emission spectrum). Fluorochromes are characterized by these two spectrums.

Jablonski diagrams may be used to explain and detail the processes of fluorochrome excitation and emission (first proposed by Alexander Jablonski in 1935) [1]; figure 1 shows a simplified diagram of this type. The vertical axis represents the fluorochrome’s energy level (in an arbitrary scale), where the ground state S (also known as fundamental state) is the least energetic one. When the fluorochrome is stimulated by electromagnetic radiation within the excitation spectrum, a transition to the excited singlet state S* may occur, from where it can return to S (by photon emission) or cross to the excited triplet state T* (by radiationless intersystem crossing). If the fluorophore is stimulated with photons that do not belong to the excitation spectrum, it will not be raised to S*. A transition from S* to S produces a fluorescent photon while a phosphorescent photon will be emitted if the transition occurs from T* to S. Emitted photons lie within the emission spectrum and are less energetic than excitation photons since they have longer wavelengths.
Figure 1. Simplified Jablonski diagram. The energy level (in arbitrary units) is shown on the left. Three different states are shown: ground S, excited singlet S* and excited triplet T*. Numbered arrows indicate transitions. Arrow 1 corresponds to fluorochrome excitation while radiationless intersystem crossing is represented by arrow 3. Arrows 2 and 4 are the only radiative transitions (fluorescent and phosphorescent photon emission, respectively).

Two terms associated with the processes of fluorescent excitation and emission are “decay” and “photobleaching”; these must not be confused [2]. Fluorescent decay involves the transition of fluorochromes in high-energy excited states to lower-energy states, from where they can be excited again. This process may involve photon emission (fluorescent or phosphorescent, represented by arrows 2 and 4 in figure 1), radiationless internal conversions and radiationless intersystem crossing (exemplified by arrow 3 in figure 1); it must be noted that this process may or may not involve the decomposition of the fluorochrome. On the other hand, photobleaching is the photo-induced chemical destruction of fluorochromes stimulated by excitation radiation. Molecules in excited states S* or T* permanently lose their fluorescent capacity due to this dynamic process, thus irreversibly reducing the total amount of fluorochromes in ground state S which may be excited.

Photobleaching is an unwanted secondary effect, since it diminishes the amount of available fluorochromes and, therefore, eliminates potentially useful information. However, certain specialized techniques take advantage of this phenomenon, such as FRAP (Fluorescence Recovery After Photobleaching, also known as Fluorescence Photobleaching Recovery) [3] and pFRET (photobleaching Fluorescence Resonance Energy Transfer) [4]. FRAP evaluates mobility or translational dynamics based on the analysis of fluorescent recovery kinetics post photobleaching, while molecular proximity measurements can be made using pFRET.

Various factors influence photobleaching. The presence of oxygen in the sample under study significantly increases the rate of photobleaching (photo-oxidation phenomenon) [2][5]. Excitation radiation intensity is another factor that must be taken into account, since high intensities imply high rates of photobleaching [6]. Additionally, the chemical microenvironment in which the fluorochrome is immersed and the chemical bonds between fluorochromes and target molecules affect the mentioned process [2][7].

It is important to take experimental conditions into consideration, since the photobleaching-analysis results of fluorochromes in solution may not be readily applied to biological specimens. This is due to the differences in the chemical microenvironment. The most common example of this difference is that fluorochromes in solution may move freely in the medium and, therefore, fluorescent molecules that have not been affected by photobleaching and photobleached fluorophores diffuse until equilibrating their relative concentrations throughout the medium. This leads to fluctuations in fluorescent levels which can not be attributed to fluorophores stimulated by the excitation beam but to the migration of molecules. Therefore, it is ideal to obtain the photobleaching curve (a curve that models this phenomenon) for the correction from samples in similar conditions to those of the samples that must be corrected.

There are several techniques to slow photobleaching down (since a method to completely prevent it from happening in wide field microscopy has not been found yet). Given that oxygen is one of the most important factors involved in photobleaching, samples may be deoxygenated by argon [2] or nitrogen [7] flushing. Moreover, anti-fading solutions (either commercial or not) may be added; substances containing N-propyl-gallate [8] are among the non-commercial ones.

There are different types of photobleaching curves, which generally present a negative exponential behavior [2][6] that is modeled by a mono-exponential or multi-exponential curve. Equations (1) and...
(2) correspond to mono- and bi-exponential curves respectively (henceforth referred to as “mono-exp” and “bi-exp”), where \( I(t) \) is intensity as a function of time \( t \), which is directly proportional to the fluorescence level. The mono-exp approach considers an homogeneous fluorochrome population with rate of photobleaching \( a \) and initial intensity \( A \), while the bi-exp models two different populations with rates \( b \) and \( c \), and initial intensities \( B \) and \( C \), respectively.

\[
I(t) = Ae^{-at} \\
I(t) = Be^{-bt} + Ce^{-ct}
\]

The main objective of the present work was to evaluate the relevance of the photobleaching curve correction. In order to do so, we used a model based on a specimen commonly used by our research group (\textit{Bufo arenarum} embryos), in which fluorescent fluctuations associated to the expression of E-cadherin was analyzed. This is a trans-membrane protein that forms adherens junctions (\textit{zonula adherens}) which help hold epithelial cells together [9][10]. Even though E-cadherin is distributed in the entire cell membrane surface, it is found in denser clusters where more than two cells meet. When this protein is immunostained with a fluorochrome (such as IgG-FITC) [11], these clusters appear brighter than the circumferential ring since it contains more molecules per unit of length; these clusters are called puncta (singular: punctum) [10].

2. Materials and methods

2.1. Photobleaching curve computation

Once the time-sequential images were obtained (see sections 2.4 and 2.5), the minimum intensity value considered as useful fluorescent signal \( F_{min} \) was established. Next, pixels in the time-zero image with value greater than or equal to \( F_{min} \) were determined (these will be referred to as “selected pixels”). Time-sequential images were then evaluated in order to acquire the fluorescence intensity value of pixels analogous to the selected ones. After averaging these values (per image), a single set that represented the temporal evolution of the average fluorescent intensity was obtained. These data were fitted using non-linear least-square regression (NLS regression, a type of curve-fitting algorithm), with an exponential model. Resulting curves were normalized to produce the photobleaching curves (figure 2).

![Figure 2](image)

**Figure 2.** Photobleaching curves. Three negative exponential curves are shown (Fit-P1, Fit-P2 and Fit-P3). These were fitted to the data extracted from selected pixels of three different histological slices (P1, P2 and P3, respectively).

2.2. Photobleaching curve correction

Pixel-intensity values were corrected employing the photobleaching curve as described below. The theoretical fluorescence value of each uncorrected image (which corresponds to a specific UV-exposure time) was located on the photobleaching curve. Next, the new fluorescence level for each pixel was obtained by dividing the uncorrected value by the one extracted from the curve (figure 3).
2.3. Quantitative evaluation

An algorithm to evaluate fluorescence recovery post photobleaching curve correction was designed in order to perform a quantitative assessment of the correction’s performance. Intensity values of selected pixels of corrected and uncorrected time-sequential images were averaged image-wise and the resulting data was fitted by NLS regression, producing average-intensity temporal evolution curves. Corrected and uncorrected curves were compared both visually and by the comparison of the rates of photobleaching of the associated equations.

2.4. Hardware and software

Digital images were obtained using a wide field microscope (Olympus BX50 Upright, 40X 0.85NA lens) equipped with a white-light source (halogen lamp) for transmitted microscopy and a mercury UV (Ultra Violet) lamp for epi-fluorescence microscopy. Images were taken with a cooled monochromatic Apogee CCD camera of 14 bits of resolution, 768x512 pixel$^2$ sensor size, 9x9 µm$^2$ pixel size, mounted to the microscope via a mount C lens (0.5X).

The CCD camera was controlled by a personal computer with an Intel Pentium II 350 MHz processor, 256 MB of RAM and 6 GB of hard-disc space, with a special software developed in our laboratory for digital deconvolution microscopy users (SUMDD) [12], to which a specialized time-lapse module for photobleaching curve correction was added. Images were automatically obtained using the SUMDD software after some basic parameters were loaded, which included (in this case) the name of the files which were to be saved, image dimensions (512x512 pixel$^2$), total amount of images (between 40 and 160), time of exposure of the CCD camera per image (1 second) and time delay between one image and the next (20 seconds). In this manner, a set of time-sequential images which represented the temporal evolution of the specimen exposed to UV-light was obtained and saved in 8-bit TIFF format.

Calculus software was used to obtain the photobleaching curve and apply the mentioned correction to a set of images. This was done in a personal computer with an AMD Athlon 64 1.8 GHz processor, 1 GB of RAM and 250 GB of hard-disc space.

2.5. Biological specimen preparation

Six histological slices were used to obtain the necessary photobleaching curves. These were produced following classic protocols [13][14] in the manner explained below.

2.5.1. Animals. Bufo arenarum tadpoles were obtained by in vitro fertilization. Adult males and females were kept in water at 20 °C for 24 hs, after which females were injected with 2500 IU of human chorionic gonadotropin (hCG) hormone (Endocorion, Elea, Buenos Aires). Twelve hours later, eggs were harvested and fertilized with testis extracts as described by Rengel et al [15]. Developmental stages were determined according to Gosner (1960) [16].

2.5.2. Histology. Stage 20 Bufo arenarum tadpoles were fixed in Carnoy solution (ethanol:chloroform:acetic acid, 6:3:1), washed in PBS (Phosphate Buffered Saline), progressively dehydrated in ethanolic solutions of increasing concentrations (Cicarelli, Buenos Aires, Argentina).
and xylol cleared (Cicarelli). Specimens were pre-included in xylol:paraffin 1:1, followed by 6 changes of paraffin (Cicarelli) and were finally included in pure paraffin. A Reichert Jung Hn 40 microtome was employed to produce transversal 5 µm-thick sections from paraffin-embedded tissues, which were mounted on 1 % gelatin-coated slides and dried at room temperature.

2.5.3. Reagents. The monoclonal antibody 5D3 is a mouse IgG1 (chicken, frog) directed to the extracellular domain of the 120 kDa isoform of the cell-cell adhesion molecule E-cadherin (Transduction Laboratories, Lexington, Kentucky, USA); it was used in a 1:50 dilution. For the detection of primary antibody binding, we used a secondary antibody (FITC-conjugated goat anti-mouse IgG; Sigma Chemical Company, St. Louis, USA) in a 1:64 dilution.

2.5.4. Immunolocalization of E-cadherin. Sections were xylol-deparaffinated, hydrated in a descending-concentration alcohol series, methanol-treated with a 0.3 % solution of hydrogen peroxide to block endogenous peroxidase for 20 minutes at room temperature, washed in PBS, treated with Triton X-100 (Sigma) 1 % for 15 minutes at room temperature and incubated in mouse normal serum 1:20 for 40 minutes at room temperature. Then, slices were incubated at 4 ºC overnight in humid chamber with primary monoclonal antibody. Next, they were washed in PBS, incubated with the secondary antibody for 2 hs at room temperature in the dark and rinsed in PBS.

3. Results

Monoexp and bi-exp curves were fitted to the sets of data extracted from selected pixels using the curve-fitting algorithm. Figure 4 shows both types of curves for the same histological slice. It is evident that the mono-exp curve does not provide a good fit, while the bi-exp one models the experimental data more precisely. A statistical analysis of all curves was performed, in which the goodness of fit was assessed by the evaluation of the coefficient of multiple determination $R^2$ (mono-exp: maximum value of 0.948; bi-exp: minimum value of 0.985) and the sum of squared errors SSE (mono-exp: minimum value of 0.01804; bi-exp: maximum value of 0.01158). Based upon the statistical results, only bi-exp curves were employed.

Figure 4. Photobleaching curves. A mono-exp $M$ ($R^2 = 0.8677$) and a bi-exp $B$ ($R^2 = 0.9919$) fit to the data extracted from selected pixels of a single histological slice (P2) are shown.

Figure 5 presents images before and after the correction. It is evident that the rapid decrease of FITC fluorescence in uncorrected images is notably reverted after the correction. As seen on the images, when fluorescence decreases, useful information is lost and contrast is reduced (since the background level diminishes less markedly) making it more difficult to distinguish genuine fluorescent signals.

In order to complement the qualitative assessment, an algorithm for the quantitative evaluation of post-correction fluorescence recovery was developed. Figure 6 shows curves that represent the temporal evolution of the average fluorescence intensity of the selected pixels of a single histological slice, before and after the correction. A distinct difference between the curves can be seen (even though both present negative bi-exponential behavior), since the uncorrected-image curve decreases more rapidly than the corrected-image one.
Figure 5. Images prior to (a-e) and post (f-j) the photobleaching curve correction. The original images were obtained from a histological slice exposed to UV-light for 1 second (a), 7 minutes (b), 14 minutes (c), 21 minutes (d) and 28 minutes (e). Bar: 18 µm.

Figure 6. Average-intensity temporal evolution curves, which were fit to the data extracted from selected pixels of a single histological slice, before and after the photobleaching curve correction (P2/Fit-P2 and cP2/Fit-cP2, respectively).

4. Discussion and conclusions
In the present work, photobleaching curve correction was evaluated in a qualitative (by simple visual inspection) and quantitative manner (with a specialized algorithm that evaluates fluorescence recovery post photobleaching curve correction).

Fluorescence-level reduction as time goes by, that is to say, as UV-exposure time increases, is mainly due to the photobleaching of FITC molecules. Since the background level diminishes less markedly than the one of FITC, it is relatively more difficult to distinguish useful fluorescent signals in images that have been exposed to UV-light for long periods of time (figure 5).

It is important to use only information extracted from selected pixels (those which at time zero have an intensity value within the range considered as useful fluorescence), since these represent the molecules of FITC that undergo the photobleaching process. Given that background autofluorescence
does not have the same photobleaching rate (mainly because it is not due to FITC), using pixels which are not selected ones would lead to an erroneous correction, since it would not consider a population made up entirely of FITC molecules.

The mono-exp curve model was discarded and, hence, the bi-exp one was adopted because, for the experimental conditions used, the mono-exp curve modeled the data less precisely (figure 4). The better fit achieved with the bi-exp curve suggests the coexistence of two populations of FITC or the presence of other fluorochromes with an emission spectrum that overlaps with the fluorescence range of interest.

Image intensity is notably recovered after applying the photobleaching curve correction (figure 5 and 6). This leads to an improvement in contrast as well as the partial recovery of information lost due to photobleaching. This type of correction presents an inherent round-off error, since the calculated new fluorescent value will not always be a whole number (integer), which must be rounded-off in order to obtain a valid pixel-intensity value. The maximum possible error is 0.39 % (1 unit in the 0-255 range), hence, it is negligible.

It is interesting to note that it takes few minutes to obtain the photobleaching curve and apply the correction to a group of 160 images; this demonstrates the celerity of the method under study.

In conclusion, we have demonstrated that the photobleaching curve correction is a relatively fast, inexpensive and simple method, which is useful for quantitative experiments since it recovers information lost due to photobleaching to a great extent.

In future works, we will evaluate variations in the photobleaching curve due to modifications in the experimental conditions (of the specimen and/or in the image-capture process). For instance, a different objective lens could be used; this would cause a variation in excitation-light intensity (even if the light source remains unchanged), since greater numerical aperture implies less excitation-light dispersion and therefore, augmented intensity. Additionally, we consider it useful to compare and contrast images corrected using the present method with images obtained from specimens immersed in different anti-fading solutions. Finally, it would be relevant to relate the bi-exp model parameters with the concentrations of the diverse populations of fluorochromes so as to fully describe the model.

References
[1] Lichtman J W and Conchello J A 2005 Fluorescence microscopy Nature Methods 2 910-9.
[2] Song L, Hennink E J, Young T and Tanke H J 1995 Photobleaching kinetics of fluorescein in quantitative fluorescence microscopy Biophysical Journal 68 2588-600.
[3] Soumpasis D M 1983 Theoretical analysis of fluorescence photobleaching recovery experiments Biophysical Journal 41 95-7.
[4] Szabo G Jr, Pine P S, Weaver J L, Kasari M and Aszalos A 1992 Epitope mapping by photobleaching fluorescence resonance energy transfer measurements using a laser scanning microscope system Biophysical Journal 61 661-70.
[5] Benson D M, Bryan J, Plant A L, Gotto A M and Smith L C 1985 Digital imaging fluorescence microscopy: spatial heterogeneity of photobleaching rate constants in individual cells The Journal of Cell Biology 100 1309-23.
[6] Marcu L, Grundfest W S and Maarek J M I 1999 Photobleaching of arterial fluorescent compounds: characterization of elastin, collagen and cholesterol time-resolved spectra during prolonged ultraviolet irradiation Photochemistry and Photobiology 69 713-21.
[7] Ma J, Chen J Y, Guo J, Wang C C, Yang W L, Xu L and Wang P N 2006 Photostability of thiol-capped CdTe quantum dots in living cells: the effect of photo-oxidation Institute of Physics Publishing, Nanotechnology 17 2083-9.
[8] Hashimoto H, Ishikawa H and Kusakabe M 1999 Preparation of whole mounts and thick sections for confocal microscopy Methods Enzymol 307 84-107.
[9] Adams C L, Chen Y T, Smith S J and Nelson W J 1998 Mechanisms of epithelial cell–cell adhesion and cell compaction revealed by high-resolution tracking of E-Cadherin–Green Fluorescent Protein The Journal of Cell Biology 142 1105-19.
[10] Gumbiner B M 1996 Cell adhesion: the molecular basis of tissue architecture and morphogenesis *Cell* **84** 345-57.

[11] Shaner N C, Steinbach P A and Tsien R 2005 A guide to choosing fluorescent proteins *Nature Methods* **2** 905-9.

[12] Diaz-Zamboni J E 2004 Software para usuarios de microscopía de desconvolución digital *Bioengineering Degree Thesis* (School of Engineering – Bioengineering, National University of Entre Ríos (UNER). Ruta 11, Km 10 (3101), Oro Verde, Entre Ríos, Argentina).

[13] Izaguirre M F, Adur J F, Peralta-Soler A and Casco V H 2001 Alterations induced by E-cadherin and β-catenin antibodies during the development of Bufo arenarum (Anura-Bufonidae) *Histology and Histopathology* **16** 1097-106.

[14] Paz D A, Alonso D G, Pisano A and Casco V H 1995 Expression of isoforms of the neural cell adhesion molecule (NCAM) and polysialic acid during the development of the Bufo Arenarum olfactory system *The International Journal of Developmental Biology* **39** 1005-13.

[15] Rengel D, Paz D, Pisano A and Pasina L 1988 Developmental capacity of Bufo arenarum aged ovocytes *Microscopia Electrónica y Biología Celular* **10** 101-13.

[16] Gosner K L 1960 A simplified table for staging anuran embryos and larvae, with notes on identification *Herpetologica* **16** 183-90.