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Fixation alters fluorescence lifetime and anisotropy of cells expressing EYFP-tagged serotonin$_{1A}$ receptor

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ABSTRACT

Fluorescence microscopic approaches represent powerful techniques to monitor molecular interactions in the cellular milieu. Measurements of fluorescence lifetime and anisotropy enjoy considerable popularity in this context. These measurements are often performed on live as well as fixed cells. We report here that formaldehyde-induced cell fixation introduces heterogeneities in the fluorescence emission of serotonin$_{1A}$ receptors tagged to enhanced yellow fluorescent protein, and alters fluorescence lifetime and anisotropy significantly. To the best of our knowledge, our results constitute the first report on the effect of formaldehyde fixation on fluorescence parameters of cellular proteins. We conclude that fluorescence parameters derived from fixed cells should be interpreted with caution.

1. Introduction

Fluorescence microscopy provides a sensitive window into molecular interactions and dynamics that are essential for cellular function [1–3]. In particular, fluorescence lifetime and anisotropy measurements represent robust approaches to monitor molecular interactions on the nanometer scale (typically 1–10 nm) by means of fluorescence resonance energy transfer (FRET)[4–6]. The presence of energy transfer is indicated by a reduction in the fluorescence lifetime of the donor in presence of acceptor in case of hetero-FRET. In contrast to hetero-FRET, homo-FRET takes place between like fluorophores and is manifested by reduction in fluorescence anisotropy [7].

The G-protein coupled receptor (GPCR) superfamily is the largest and most diverse protein family in mammals, involved in signal transduction across membranes [8]. They represent major targets for the development of novel drug candidates in all clinical areas [9].The serotonin$_{1A}$ (5-HT$_{1A}$) receptor is an important member of the GPCR superfamily and is the most extensively studied among the serotonin receptors for a variety of reasons [10].The serotonin$_{1A}$ receptor agonists and antagonists have been shown to possess potential therapeutic effects in anxiety-or stress-related disorders. As a result, the serotonin$_{1A}$ receptor serves as an important target in the development of therapeutic agents for neuropsychiatric disorders such as anxiety and depression.

Fixation is a commonly employed procedure in cell biology that renders cells permeable to stains and allows cross-linking of cellular macromolecules so that their spatial distribution is stabilized. This allows better visualization for longer period of time. Although fixation is an extensively used technique, its effect on fluorescence microscopic parameters (such as fluorescence lifetime and anisotropy) is rarely explored. This is unfortunate since fluorescence parameters obtained from microscopic studies are often reported and compared in the literature, irrespective of treatments such as fixation. We report here the effect of fixation on fluorescence lifetime and anisotropy of EYFP-tagged serotonin$_{1A}$ receptor (5-HT$_{1A}$R-EYFP) heterologously expressed in CHO cells. To the best of our knowledge, our results constitute the first report on the effect of formaldehyde fixation on fluorescence parameters of cellular proteins.

2. Material and methods

2.1. Materials

$MgCl_2$, $CaCl_2$, penicillin, streptomycin and gentamicin sulfate were obtained from Sigma (St. Louis, MO, USA). D-MEM/F-12 [Dulbecco's modified Eagle medium: nutrient mixture F-12 (Ham) (1:1)], fetal calf serum, and geneticin (G 418) were from Invitrogen Life Technologies (Carlsbad, CA, USA). All other chemicals used were of the highest available purity. Water was purified

Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); 5-HT$_{1A}$R-EYFP, 5-hydroxytryptamine$_{1A}$ receptor tagged to EYFP; EYFP, enhanced yellow fluorescent protein; FLIM, fluorescence lifetime imaging microscopy; FRET, fluorescence resonance energy transfer; GPCR, G-protein coupled receptor.

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through a Millipore (Bedford, MA, USA) Milli-Q system and used throughout.

2.2. Cells and cell culture

CHO-K1 cells stably expressing the serotonin$_{1A}$ receptor tagged to enhanced yellow fluorescent protein (referred to as CHO-5-HT$_{1A}$R-EYFP) were used ($\sim 10^5$ receptors/cell). Cells were grown in D-MEM/F-12 (1:1) supplemented with 2.4 g/l of sodium bicarbonate, 10% fetal calf serum, 60 μg/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamicin sulfate in a humidified atmosphere with 5% CO_2 at 37 °C. CHO-5-HT$_{1A}$R-EYFP cells were maintained in the above-mentioned conditions with 300 μg/ml geneticin. Cells were fixed with 3.5% (v/v) formaldehyde for 10 min.

2.3. Fluorescence lifetime imaging microscopy (FLIM)

FLIM experiments were carried out using a lifetime imaging attachment (Lifetime Imaging Fluorescence Attachment (LIFA), Lambert Instruments, Leutingwolde, The Netherlands) mounted on an inverted microscope (TE2000U, Nikon, Japan). The tagged receptor was excited using epi-illumination with a sinusoidally-modulated 470 nm LED at 40 MHz and observed with a 100× (NA 1.25) oil objective (Nikon Plan-Fluor, Nikon, Japan) through a filter set (Nikon FITC, DM505, EM 515–555 nm). The phase and modulation lifetimes were determined from a series of images taken at twelve phase settings using software provided by the manufacturer. A pseudo random recording order provided by the software was used to correct for any photobleaching [11]. Rhodamine 6G in distilled water (lifetime 4.1 ns) was used as a reference [12].We averaged the FLIM data on a cell-to-cell basis since we are interested in the statistics of a cell population.

2.4. Fluorescence anisotropy imaging microscopy

Anisotropy imaging experiments were carried out using the same set-up as described for the FLIM experiments with the inclusion of a polarizer in the excitation path of the microscope and a dual-view polarizing beam splitter in the emission path prior to the LIFA intensifier-CCD camera. CHO-5-HT$_{1A}$R-EYFP cells were excited using epi-illumination with a 470 nm LED at constant illumination (unmodulated) and observed with a 100× (NA 1.25) oil objective, using Nikon FITC filter, as described above. The parallel and perpendicular components of the emission were imaged synchronously on both halves of the CCD chip. Images were corrected for instrumental differences in the detection of parallel and perpendicular components of emission by using Rhodamine 6G in distilled water as a reference and an anisotropy of 0.012, measured in a Varian Eclipse spectrofluorimeter, as described previously [2]. In order to correct for the depolarization caused by the high aperture objective used in this study, Rhodamine 6G was taken in solutions of varying glycerol content and anisotropies were simultaneously measured under the microscope and the fluorimeter, and a calibration plot was generated. The correction for aperture depolarization in the microscope was done by multiplying the anisotropy determined under the microscope with a constant factor of 1.47, derived from the slope of the calibration plot.

Individual cells were selected using region-of-interest tools in ImageJ (NIH, Bethesda, USA) and the parallel (I_{par}) and perpendicular (I_{perp}) intensity values were analyzed. The total fluorescence was given by:

$$I = I_{par} + 2GI_{perp}$$

where G is the correction factor for instrumental differences in detection in the parallel and perpendicular halves of the CCD. The mean anisotropy is given by:

$$r = (I_{par} - GI_{perp})/I$$

Image arithmetic was performed in ImageJ (NIH, Bethesda, USA).Plotting and analysis was carried out using Origin software version 7.0 (OriginLab Corp., Northampton, MA, USA) and Microsoft Excel 2007.

3. Results and discussion

We have previously pharmacologically characterized the heterologously expressed serotonin$_{1A}$ receptor tagged to EYFP in CHO cells (termed 5-HT$_{1A}$R-EYFP) and shown that the tagged receptors are essentially similar to the native receptor [13].The average phase- and modulation-detected lifetimes of 5-HT$_{1A}$R-EYFP in cell membranes are shown in Table 1. In live cells, i.e., in the absence of fixation, the phase lifetime (τ_Φ) was ~3.6 ns and the modulation lifetime (τ_M) was ~3.7 ns. These lifetime values are comparable to those measured for EYFP in solution [14,15] and other EYFP-tagged proteins in cells [16]. The close agreement between the phase and modulation lifetimes suggests an overall homogeneous environment experienced by the EYFP fluorophore inside the cell [16,17]. This is also consistent with the reported largely single exponential decay behavior of this fluorophore [14]. Table 1 shows that formaldehyde fixation alters the lifetime values of 5-HT$_{1A}$R-EYFP. The phase and modulation lifetimes are reduced to ~2.9 and ~3.5 ns upon fixation. The relatively large change in phase lifetime indicates that phase lifetime is more sensitive. It should be noted here that a trivial change in the environment of EYFP, possibly induced by fixation (such as a change in refractive index) would not show this difference since in that case both the phase and modulation lifetimes would be equally affected. The lack of agreement between the phase and modulation lifetimes in case of fixed cells could be due to multi-exponential decay of fluorescence. It is possible that fixation induces environmental heterogeneity around the fluorophore, leading to multi-exponential decay. For a population of fluorophores exhibiting multi-exponential decay, the shorter decay times are more heavily weighted and therefore results in a lower value of the apparent phase lifetime [17]. It is also possible that fixation induced dehydration of cells could lead to the formation of microheterogeneous environments around the fluorophore leading to the heterogeneity in fluorescence lifetime [18,19]. Representative lifetime images of live and fixed cells are shown in Figs. 1 and 2, along with the corresponding intensity distributions. Interestingly, fixation does not produce any perceptible change in spatial distribution of the tagged receptor (panel A in Figs. 1 and 2) or lifetimes (panels B and C in Figs. 1 and 2).

The anisotropy values of 5-HT$_{1A}$R-EYFP in live and fixed cells, are shown in Table 2. Fixation appears to have a drastic effect on the fluorescence anisotropy of 5-HT$_{1A}$R-EYFP. While the anisotropy in live cells was ~0.22, it increased to ~0.36 in fixed cells. This is a large increase, keeping in mind the anisotropy scale. This increase in anisotropy could be due to the fact that fixation induces cross-linking of cellular proteins, which could have two possible effects:

Table 1
Average fluorescence lifetime of 5-HT$_{1A}$R-EYFP in CHO cells[a].

Condition	Phase lifetime (τ_Φ) (ns)	Modulation lifetime (τ_M) (ns)	N
Live cells	3.55 ± 0.09	3.69 ± 0.05	30
Fixed cells	2.85 ± 0.06	3.46 ± 0.12	44

N represents the number of cells over which measurements were carried out. Means ± standard deviations are shown. Values for live cells are from Ref. [20]. See Section 2 for other details.

[a] Excitation at 470 nm with LED; emission collected between 515–555 nm.

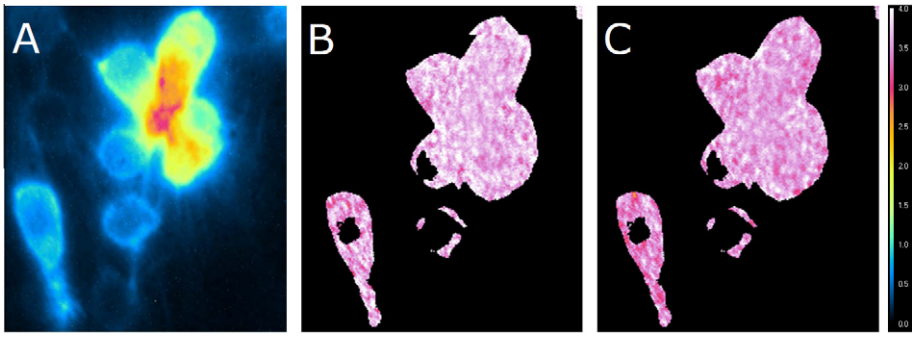

Fig. 1. Frequency domain fluorescence lifetime imaging microscopy (FLIM) of live CHO-5-HT_{1A}R-EYFP cells. The intensity image (blue: low, white: high) is shown in panel (A). The corresponding modulation and phase lifetime images (color coded in a scale of 0–4 ns) are shown in panels (B) and (C), respectively. Images represent the spatial distribution of fluorescence intensity, modulation and phase lifetimes of 5-HT_{1A}R-EYFP. Note the similarity in phase and modulation lifetime images. Images shown are adapted from Ref. [20]. See Section 2 for other details. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

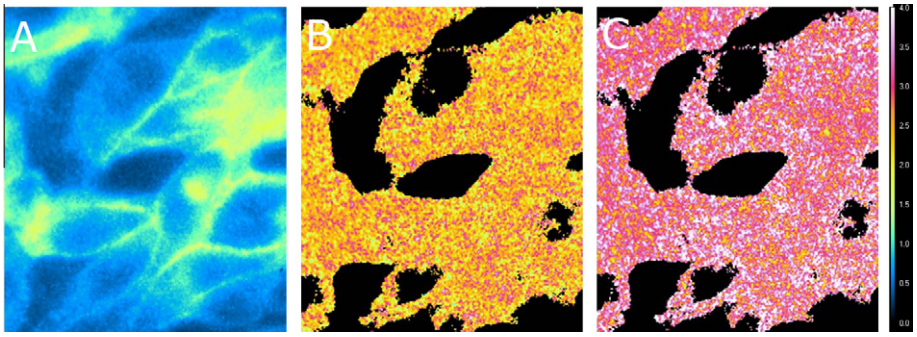

Fig. 2. Frequency domain fluorescence lifetime imaging microscopy (FLIM) of CHO-5-HT_{1A}R-EYFP cells fixed with 3.5% (v/v) formaldehyde. The intensity image (blue: low, white: high) is shown in panel (A). The corresponding phase and modulation lifetime images (color coded in a scale of 0–4 ns) are shown in panels (B) and (C), respectively. Images represent the spatial distribution of fluorescence intensity, modulation and phase lifetimes of 5-HT_{1A}R-EYFP. The difference between phase and modulation lifetimes in these images are apparent under these conditions. See Section 2 for other details. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Average anisotropy for 5-HT_{1A}R-EYFP in CHO cells[b].

Condition	<r>	*N*
Live cells	0.223 ± 0.024	8
Fixed cells	0.361 ± 0.05	20

N represents the number of cells over which measurements were carried out. Means ± standard deviations are shown. Values for live cells are from Ref. [20]. See Section 2 for other details.

[b] Excitation at 470 nm with LED; emission collected between 515–555 nm.

(i) fixation-induced reduction of rotational mobility, which would increase the anisotropy, and (ii) cross-linking of 5-HT_{1A}R-EYFP to an unlabeled membrane protein that would result in a decrease in the extent of homo-FRET and would also increase anisotropy. Photoinduced processes leading to a slower rotational motion appears unlikely since the increased anisotropy is only observed in fixed cells. Alternately, fixation may introduce new fluorescent species that could have a large intrinsic anisotropy. In summary, our results show that formaldehyde-induced cell fixation introduces heterogeneities in the fluorescence emission of 5-HT_{1A}R-EYFP and complicates interpretation of lifetime and anisotropy measurements. We recommend that caution should be exercised while interpreting fluorescence parameters derived from fixed cells.

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