Biochimica et Biophysica Acta 1863 (2016) 322–334

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Depolarized FRET (depolFRET) on the cell surface: FRET control by photoselection

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ARTICLE INFO

Article history:
Received 15 September 2015
Received in revised form 23 November 2015
Accepted 2 December 2015
Available online 3 December 2015

Keywords:
Polarizationally structured light
Circularly polarized light
Diagonal-polarization
CORNU-depolarizer
Orientation factor for FRET
Homo-FRET

ABSTRACT

Sensitivity of FRET in hetero- and homo-FRET systems on the photoselected orientation distribution of donors has been proven by using polarized and depolarized light for excitation. FRET as well as donor and acceptor anisotropies have been simultaneously measured in a dual emission–polarization scheme realized in a conventional flow cytometer by using single laser excitation and applying fluorophore-conjugated mAbs against the MHCI and MHCII cell surface receptors. Depolarization of the originally polarized light have been achieved by using crystal depolarizers based on Cornu's principle, a quarter-wave plate for circular polarization, and a parallel beam splitter acting as a diagonal-polarizer for dual-polarization excitation. Simultaneous analysis of intensity-based FRET efficiency and acceptor depolarization equivocally report that depolarization of light may increase FRET in an amount depending on the acceptor-to-donor concentration ratio. Acceptor depolarization turned to be more sensitive to FRET than donor hyper-polarization and even than intensity-based FRET efficiency. It can be used as a sensitive tool for monitoring changes in the dynamics of the donor–acceptor pairs. The basic observations of FRET enhancement and increased acceptor depolarization obtained for hetero-FRET are paralleled by analog observations of homo-FRET enhancements under depolarized excitation. In terms of the orientation factor for FRET, the FRET enhancements on depolarization in the condition of the macroscopically isotropic orientation distributions such as those of the cell surface bound fluorophores report on the presence of local orientation mismatches of the donor and acceptor preventing the optimal FRET in the polarized case, which may be eliminated by the excitation depolarization. A theory of fluorescence anisotropy for depolarized excitation is also presented.

1. Introduction

Fluorescence resonance energy transfer (FRET) is a highly sensitive and versatile tool for monitoring orientation and distance changes of fluorophores located at 1–10 nm separations. Due to this attribute, FRET is extensively used for monitoring conformational changes and proximities of biological macromolecules, even in these days in the era of super resolution microscopes. For efficient FRET between a pair of fluorophores, essentially 3 main requirements should be fulfilled: (i) Large overlap between the spectra of donor emission and acceptor absorption on the wavelength scale, (ii) small enough distance between the fluorophores dictated by the above overlap, and (iii) favorable orientation between the fluorophores formulated by the constraint called "orientation factor for FRET" (κ^2) [1–5]. Because the subject of this work is the exploitation of this orientation constraint for a more optimized FRET detection, and because excellent literature is available for FRET theory, we explain only this last parameter in more detail. Without going into quantitative details, the orientation factor expresses the fact that FRET for a given donor–acceptor separation and spectral overlap is favorable only for certain relative orientations of the donor and acceptor dipoles and their joining line, and unfavorable for the others, with the consequence that, for a given donor orientation FRET proceeds only towards those acceptors having the favorable orientations [6–8]. This also means that after the 1st photoselection process of creating the orientation distribution of the excited donors by the exciting light, there exists a 2nd photoselection process of creating the orientation distribution of the excited acceptors by the donor's local electric field [10–14]. Due to this 2nd photoselection, the orientation distribution of the FRET-excited acceptors may be affected by the orientation distribution of donors, i.e. FRET may happen only towards those acceptors having proper orientations. This property of FRET can also be conceived as an

Abbreviations: FRET, fluorescence resonance energy transfer; MHCI/MHCII, Class I/Class II Major Histocompatibility Complex proteins I and II; β_2m, beta-2 microglobulin, the light chain (l.c.) component of MHCI; mAb, monoclonal antibody.

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http://dx.doi.org/10.1016/j.bbamcr.2015.12.003
0167-4889/

orientation switch for the migrating excitation energy. In the past, this property have been exploited by several researchers for optimizing FRET detection: creating FRET between certain donor and acceptor dyes, one of which can be considered as an isotropic emitter or absorber, e.g. lanthanides with degenerate transitions, phycobiliproteins (Phycoerythrin) with tandem dyes, and quantum dots (QDs) [8,12,13].

The orientational optimization of FRET – i.e. making the FRET process independent from the relative donor–acceptor orientations – in these schemes is on the single fluorophore level, by choosing isotropic donors and/or acceptors. However, similar optimization of FRET can also be carried out on the ensemble level of the primarily photoselected donor fluorophores. An indication for that this may be possible can be found in the elegant paper of Corry et al. [15], who have clearly shown that membrane bound dyes may have anisotropic local orientation distributions, in spite of the fact that, the whole ensemble of fluorophores can be considered as an isotropically distributed one. They also nicely demonstrate that this local anisotropy manifests itself in anisotropic absorption of light due to photoselection, which can be utilized for determining limits on the orientation factor for FRET, the κ^2. Taking into account that FRET depends on the relative donor–acceptor orientations ("2nd photoselection") similarly to the way absorption depends on the relative photon field-dye orientations, the implication of these observations is that, not only the absorption process, but also the FRET process may be sensitive to the anisotropy of orientation distributions in polarized exciting light. As a closer inspection, taking now two anisotropically distributed types of dyes – the donors and the acceptors – bound to membrane receptors, it is expected that for polarized excitation only for donor–acceptor dyes pairs found in specific locations of the cell membrane will FRET be optimal (Fig. 1) [15,16]. In contrast, for depolarized excitation each position in the cell membrane can be taken as equivalent concerning FRET, an indication for an enhanced sensitivity of FRET detection in depolarized exciting light.

For achieving a more complete description of the FRET effects of depolarized excitation a dual-polarization detection scheme ("Single laser polFRET") has been applied in a flow cytometer, where in addition to the FRET efficiency, donor and acceptor anisotropies are also detected on a cell-by-cell basis [17]. Hyper-polarization of donor anisotropy is expected whenever FRET efficiency increases, due to the lifetime shortening of the donors. On the acceptor side, however, a reduction of anisotropy is expected due to the depolarized excitation by FRET. Although both anisotropies change with FRET, acceptor anisotropy is expected to be more sensitive than the donor anisotropy. While the

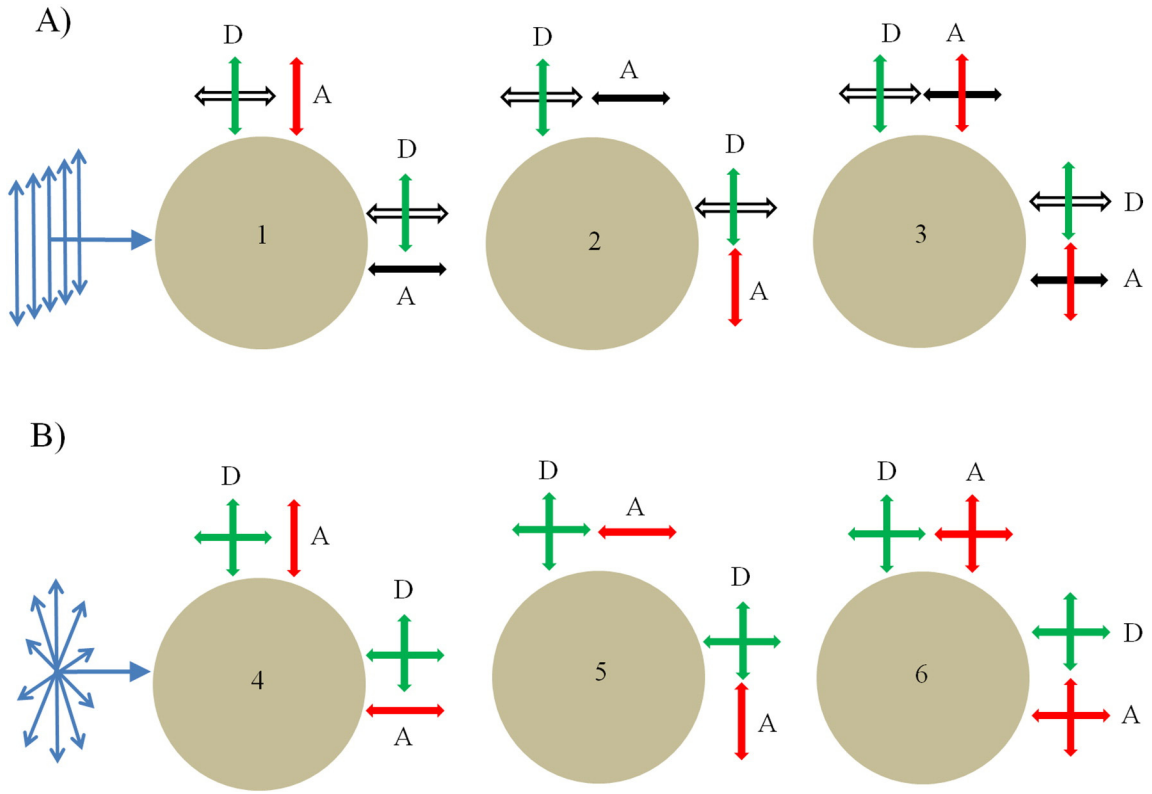

Fig. 1. *Cartoon illustrating FRET enhancement by excitation depolarization*. Due to the orientational restriction of FRET formulated in the "orientation factor", excitation with depolarized light can be more favorable for FRET than excitation with polarized one, by the fact that in depolarized light more donor orientations are sampled. The magnitude of the expected FRET enhancement is governed by factors such as the degree of orientation randomness, rotational dynamics, and the relative abundance of the donor and acceptor. Orientation distribution of dyes targeted through ligands to membrane receptors – such as the Alexa-labeled receptors in our case – can be substantially anisotropic and their rotational motion highly restricted favoring the occurrence of the FRET enhancement effect. Besides receptor-tethered dyes and VFPs, lipid-intercalating dyes such as DiI, Bodipy PC, and DPH having strongly anisotropic orientation distributions can also be the candidates to show this effect. Illustrated is that, for anisotropic acceptor orientation distributions (relative to the cell membrane) in polarized light spatial inhomogeneities of FRET can occur on the cell membrane, which can be eliminated by depolarization of the exciting light (blue arrows on the left). Orientation distributions of the donors and acceptors relative to the cell membrane are represented by the double headed arrows positioned to the north pole and to the equator of the cells represented by gray filled circles (numbered #1–#6). Green filled and empty double headed arrows represent excited and non-excited donors, red and black filled ones represent excited and non-excited acceptors. The orientation distributions and the orientation dependence of FRET are represented by strongly simplified manners. All dipoles can assume only two orientations: either parallel or perpendicular to the local membrane plane. On all cells, the donors are randomly distributed, represented by crossed double arrows. Acceptors are oriented either perpendicular to the membrane plane (cells #1, #4), parallel (cells #2, #5), or randomly (cells #3, #6). FRET is conceived as a two-state switch: it can occur only between parallel donor and acceptor dipoles ("closed switch"), and cannot occur between perpendicular ones ("open switch"). *Row A*: Excitation by vertically polarized light. From the isotropically oriented donors only the vertical ones are photoselected. Due to the orientation restriction of FRET, while for the perpendicular and parallel orientations of the acceptor only acceptors located on the north pole (cell #1) or the equator (cell #2) of the cells can take part in FRET, for isotropic acceptor orientations (cell #3) in both membrane locations there is some FRET. *Row B*: In depolarized light no spatial FRET inhomogeneity exists (cells #4–#6), regardless of the orientation distribution of the acceptor.