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Confocal microscopic dual-laser dual-polarization FRET (2polFRET) at the acceptor side for correlating rotations at different distances on the cell surface

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ABSTRACT

Relationship of donor and acceptor fluorescence anisotropies as well as efficiency of fluorescence resonance energy transfer (FRET) has been investigated in a confocal microscope in the context of FRET systems comprised of donor and acceptor-labeled MHCI and MHCII receptors on the surface of Kit-225 K6 human T-cells. The measurements have been carried out in a 2-laser, 5-signal platform where the total donor fluorescence intensity and 2 acceptor fluorescence intensities with their anisotropies – one at the donor's excitation wavelength, the other at the acceptor's excitation wavelength – have been detected. This configuration enabled the determination of FRET efficiency and correlating it with the two acceptor fluorescence anisotropies as a kind of calibration. Estimations for the FRET-enhanced donor fluorescence anisotropy, the directly excited acceptor fluorescence anisotropy, and the fluorescence anisotropy of sensitized emission have been obtained. Procedures for determining FRET by measuring only the total donor intensity and the acceptor intensity and its anisotropy, or two acceptor intensities and their anisotropies have been elaborated, the errors of which have been estimated based on the fluorescence anisotropy values obtained in the calibration with the method of flow cytometric energy transfer (FCET).

The combined detection of the donor and acceptor fluorescence anisotropies enabled also the determination of the lower and upper limits of the orientation factor for FRET (κ^2). An increase in range for κ^2 with increasing FRET efficiency has been observed, with average κ^2 values different from the dynamic random average of 2/3. These observations call for the need of κ^2 determination in proximity measurements, where the donor and acceptor orientations are not predictable.

An increasing range of κ^2 with increasing intermolecular proximity of the MHCI and MHCII receptors has been observed. This indicates that molecular flexibility in the clusters of the MHCI and MHCII receptors reduces with increasing cluster density, i.e. a “fluidity gradient” exists in the clusters. More specifically, the local density dependent flexibility can also be taken as a direct proof for that the association of these receptors is non-random, but mediated by some type of physical interaction, a finding as a benefit of FRET detection by polarization spectroscopy.

Two new quantities – the quenched donor fluorescence anisotropy and a fluorescence anisotropy analogue, the “dissymmetry index” of the polarized FRET efficiency components – have also been introduced for the characterization of the orientational dynamics of the excited state during FRET.

1. Introduction

Even in the era of super-resolution microscopies fluorescence resonance energy transfer (FRET) remains the leading technology for detecting molecular proximity in the 1–10 nm distance range even on the surface or inside the living cell. This outstanding property of FRET rests on its inverse sixth power distance dependence, described by the Förster's formula for the FRET rate constant ($k_t \propto R^{-6}$, where k_t is the rate constant for FRET, and R is the average separation of the donor and acceptor dyes) [1].

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

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The detection of FRET – based on the exploitation of some photophysical manifestation of FRET – is very wide-ranging [1–5]. It reaches from the measurement of simple donor quenching and sensitized acceptor emission [2–5] to the more sophisticated measurement of photobleaching like lengthening of photobleaching time of the donor (pbFRET) [2,6,7], shortening of photobleaching time of the acceptor by FRET [8] and FRET-frustration by acceptor-photobleaching (apbFRET) [2,9] or by saturation of acceptor (satFRET) [10]. Besides, FRET can also be detected via the different direct fluorescence lifetime measuring (FLIM) techniques [11–13].

In the past, we and others successfully used a flow cytometric technique called “dual laser flow cytometric FRET” (FCET) for detecting receptor proximity maps on the cell surface [14–19]. In the scheme of FCET, the three unknown FRET parameters – the FRET efficiency and the donor and acceptor concentrations – are characterized by three measured signals, the quenched donor and sensitized acceptor fluorescence intensities (I_1, I_2) both measured at the donor's excitation wavelength, and from the 3rd fluorescence signal (I_3), measured in the acceptor's emission channel at its excitation wavelength. Amongst of these 3 signals, I_1 and I_2 are the most sensitive to FRET, and I_3 has only a modest sensitivity, describing mostly the directly excited acceptor fluorescence.

Although FCET is successful as it is, a shortcoming of the method is that it gives no account of the donor and acceptor dipole orientations prevailing during FRET, i.e. it gives no information on the FRET orientation factor (κ^2) vital for translating FRET efficiencies into molecular proximities [20–23]. To circumvent this shortcoming, as an “upgrade of FCET”, the 3polFRET method has been introduced in a flow cytometer by detecting the polarized components of the 3 fluorescence intensities I_1, I_2, I_3 [24], or equivalently 3 fluorescence anisotropies and the corresponding 3 total intensities.

Besides estimating the limits of κ^2, 3polFRET enables the determination of associated donor fraction for high-enough signal levels and estimation of the rotational parameters describing molecular dynamics and ultimately the correlation of all of these 3 parameters with the FRET efficiency. Phenomenologically, the increased capability of 3polFRET rests on two additional photophysical effects of FRET – besides the more conventional donor quenching and sensitized emission exploited in FCET and in other techniques – the enhancement of donor fluorescence anisotropy due to shortening of time available for rotation i.e. the fluorescence lifetime of donor, and the depolarization of acceptor fluorescence anisotropy due to depolarized excitation by the curvy donor dipole field (the 2nd photoselection) (Fig. 1) [25–31]. As to the practical application, the importance of these phenomena lies in that, while in the absence FRET donor and acceptor fluorescence anisotropies are sensitive only to rotations, they become sensitive also to lateral motion in the presence of FRET, implying an increase in their sensitivity to molecular dynamics.

For these phenomena donors of low fluorescence anisotropy – e.g. antigen bound whole mAb, tethering a dye through a linker – but of rotational correlation time falling on the time-scale of FRET and acceptors of high fluorescence anisotropy – e.g. engineered green, yellow fluorescent protein, GFP, YFP – are favored. Because of their biological effector functions, mAbs possess considerable motional freedom – rotation, wagging and elbow bend of the Fab arm, and wagging of Fc portion [32] (see also Supporting information) – which is added to the tethering motion of the dye around its linker, which can be also substantial, depending on the length of linker. By contrast, chromophore groups of visible fluorescent proteins (VFPs) are fairly rigidly fixed in their β-barrel shields, constraining segmental flexibility to that of the holding β-barrel as a whole, which in turn is dictated by its intra-protein position, namely that whether it is in an intra-chain, or in a chain-end, C- or N-terminal, position [33–35].

In this communication we show that by some relaxation of the condition of 3polFRET in a confocal microscope, namely by detecting only the total donor intensity and the fluorescence anisotropy and

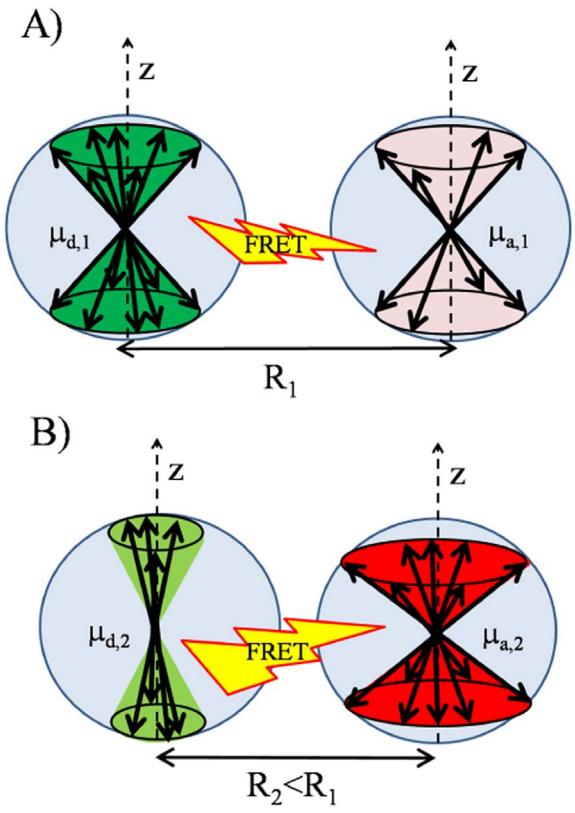

Fig. 1. Modulation of fluorescence polarization by FRET.
Ensemble level steady state orientational distributions of the excited state dipoles ($\mu_{d,1}$, $\mu_{a,1}$) of the donor and acceptor are represented by green and red double conical volumes, which are carved out from space by the tips of the respective dipole vectors (black double-arrows) with their middle points at donor-acceptor separations R_1 (Panel A) and R_2 (Panel B). Shading intensity is proportional to the number of excited state dipoles.
Panel A: The donor orientational cone represents the ensemble of those donors which have not decayed yet via fluorescence and FRET after directly exciting them by light. Similarly, the acceptor orientational cone represents the ensemble of those acceptors which have not decayed yet via fluorescence, after directly exciting them by light or indirectly by FRET. For the sake of convenience only arrows with their tips on the cone surface are shown. The orientations are represented by the angles of the arrows relative the axis of symmetry of the orientational cones which also corresponds to polarization direction of the exciting light (z-direction). Only angles in a limited range around the reference direction are populated by the arrows corresponding to anisotropic orientational distributions i.e. non-zero fluorescence anisotropies. For isotropic orientation distributions the double cones would turn into spherical volumes (light blue discs) with the same centers. For the donors (left arrows) photoselection in absorption to an excited electronic and vibronic state, then the concomitant relaxation to the lowest vibrational level of the 1st excited electronic state – according to Kasha's rule – as well as rotational Brownian motion much quicker or as fast as fluorescence are the responsible for the deviations of the individual arrows from the vertical z-axis. For the acceptor (right arrows) depolarizing effect of the indirect excitation by the curvy donor local field in FRET should be added to those – effect of photoselection, vibrational relaxation and physical rotation – prevailing after direct excitation by light. In the languge of fluorescence anisotropy, the depolarizing effect of photoselection itself is described by the $r_f = 0.4$ fundamental anisotropy, while those of vibrational relaxation and rotations much quicker than fluorescence, by the r_0 limiting anisotropy [44,45].
Panel B: At a reduced separation ($R_2 < R_1$) FRET is stronger leading to decreased brightness and narrower orientation distribution for the excited donors in the steady state (light green double cone), and increased brightness and broader orientation distribution for the excited acceptors (dark red double-cone). With increasing FRET, for the donors larger angles are depleted, smaller ones are populated. This is due to the fact that for grater FRET, the time duration available for rotation of the donor excited state dipole – i.e. the donor fluorescence lifetime – is reduced, preventing visiting grater angles from the vertical direction (z-axis). By contrast, for the acceptors smaller angles are depleted and larger ones are populated. This is due to the depolarized excitation via the donor local field in FRET manifested in parallel with the direct excitation by light. The distribution will be more anisotropic (“elongated”) for the donor and more isotropic (“spherical”) for the acceptor. The lengths of the individual donor and acceptor dipoles – and consequently, the radiative rates and intrinsic lifetimes – are not affected by FRET ($\mu_{d,1} = \mu_{d,2}$, $\mu_{a,1} = \mu_{a,2}$), except for their numbers represented also by the intensity of coloring of the orientational cones.