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Dual-laser homo-FRET on the cell surface

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ABSTRACT

Inhomogeneous broadening and red-edge effects have been detected on a highly mobile system of fluorescently conjugated mAbs targeted to cell surface receptors. By exploiting site-selective spectroscopy and the characteristic loss of homo-FRET on increasing excitation and decreasing emission wavelengths, contributions of physical rotation and homo-FRET to the depolarization of fluorescence anisotropy have been separated. Absolute homo-FRET efficiency has been determined by ratioing two anisotropies: a homo-FRET-sensitive one, which is excited at the absorption main band and detected at the long wavelength region of emission, and a homo-FRET-insensitive one, which is excited at the long wavelength region of absorption and detected at the short wavelength region of emission. Because the anisotropies are simultaneously detected in a unified detection scheme of a dual T-format arrangement, the method is applicable for the real-time tracking of dynamical changes of physical rotations and proximities. The utility of the method is demonstrated in the context of the MHCII molecule and the heavy and light chains of the MHCI molecule, a system of three receptors with well-characterized close mutual proximities. Although the method is presented for a flow cytometer, it can also be realized in a fluorescence microscope capable for dual-laser excitation and dual-anisotropy detection.

1. Introduction

Homo-energy transfer (homo-FRET) is an important phenomenon for detecting and quantifying receptor clusters in the 1–10 nm inter-receptor separation range by using a single type of fluorophore [1–9]. Generally it is measured through its depolarizing effect exerted on the fluorescence anisotropy. Because possible rotational Brownian-motion of the fluorophores could also contribute to depolarization of anisotropy, homo-FRET can most readily be detected for systems possessing highly restricted rotational mobility on the nsec time-scale, e.g., different kinds of visible fluorescent proteins (VFPs) [1,10,11]. In the more general case of fluorophores having substantial rotational mobility, a common way of separating the effects of homo-FRET and rotational motion is changing the concentration of fluorophores by either applying different amounts of dyes for labeling or by photobleaching [12,13]. The limitations of these approaches are that they require multiple samples and/or they are not reversible precluding real time monitoring of dynamic processes when both proximity and mobility can change simultaneously. Additionally photobleaching may be applied mainly in microscopy rather than flow cytometry due to the required high light doses. A reversible way of depressing homo-FRET may be absorption saturation, but this may require high illumination intensities which may interfere with life processes [14].

Aiming at the generalization of the approach set out by A. Squire et al. [10] – who measured homo-FRET between practically immobile VFP chromophores – for systems having larger degree of rotational mobility than VFP, we propose an alternative method for the isolation and the optimization of the detection of homo-FRET. It is accomplished by exploiting the characteristic wavelength dependencies of homo-FRET, i.e., the absorption red-edge and emission blue-edge effects [11, 15–20], shown in the presence of inhomogeneous broadening.

Inhomogeneous broadening is the phenomenon when the molecular energy levels become distributed due to different interaction strengths with the local environment, such as solvent shells, protein and lipid milieus. Because the energy levels of different molecules are affected differently, this broadening is called inhomogeneous, in contrast to the homogeneous broadening when the energy levels of all

Abbreviations: FRET, fluorescence resonance energy transfer; 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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molecules are affected the same fashion (e.g., vibrational- or termal-broadening, velocity- or Doppler-broadening, natural-broadening of spectra). While the deeply lying energy levels are occupied by the most strongly interacting particles, the upper lying levels are occupied by the weakly interacting ones. In the method called site photoselection, it is possible to monitor only a specified subpopulation of the total one at custom according to the strength of interaction with the environment. This is achieved with narrow-band light sources tuned in wavelength to the energy of the subpopulation in interest. Specific, environment sensitive "2-state" dyes working on these principles have developed recently for monitoring fluidity gradients in membranes, membrane surface potential, dipole potential, and lipid phase transitions ("potential and fluidity probes", "polarity dyes") [17–19]. Because FRET is governed by the spectral overlap between absorption and emission spectra, molecular rendering according to energy levels introduces directionality in FRET, implying energy migration in the direction of decreasing energy (Fig. 1, Panel A) [20]. The absorption red-edge effect – discovered by G. Weber in 1960 [16] – and the emission blue-edge effect are consequences of this general principle, and they refer to failure of FRET due to the depletion of energy acceptors and donors for FRET, respectively (Fig. 1, Panel B).

Concerning first the absorption red-edge effect, elimination of the effect of rotational depolarization may be attempted by ratioing two anisotropy values simultaneously measured at the maximum and at the long wavelength edge of the absorption spectrum (red-edge), the latter anisotropy being dependent only on rotation, while the former being dependent on both homo-FRET and rotation. The emission counterpart of this phenomenon, called the blue-edge effect, also exists: While the anisotropy detected at the maximum of the emission spectrum is affected by both rotation and homo-FRET, the one detected at the short wavelength emission edge (blue-edge) is affected by only rotation, thereby offering another possibility for the elimination of the effect of rotation by ratioing two appropriate anisotropies. By combining these two effects for increasing efficiency, we aimed to separate rotation and homo-FRET by sequentially photoselecting a subpopulation sensitive mainly to rotation, accomplished with red-edge excitation, blue-edge emission, and another one equally sensitive for both rotation and homo-FRET, accomplished with main-band excitation, red-edge emission, in a flow cytometer. Besides the spectral heterogeneity, and directionality in FRET however, heterogeneities in other spectral characteristics such as fluorescence lifetime and rotational correlation time, may also arise, which can be taken into account in a calibration procedure.

We first demonstrate the existence of the red-edge effects for the surface-tethered dyes. Then we show the feasibility of a hybrid approach which takes into account both the aforementioned red-edge and blue-edge effects for an efficient separation of the depolarizing effects of homo-FRET and rotation in flow cytometric dual-laser dual-anisotropy homo-FRET determinations in clusters of the MHCI and MHCII molecules. These are two important cell surface immune receptors vital in the initiation of T-cell mediated immune responses [4,21]. The receptors were labeled with fluorescently stained mAbs. Practical questions such as sensitivity of the method to the strength of homo-FRET, the dye's tethering motion, and segmental flexibility of the dye-docking protein moieties have been addressed by applying different types of fluorophores such as Alexa Fluor-488 (A488) and the highly mobile xFITC, a dye with a 7-atom spacer, for staining the Fab portions as well as the whole versions of mAbs at different dye/protein labeling ratios. The significance of the approach is that it enables the separation the depolarizing effects of homo-FRET and rotational motion, i.e., it enables the simultaneous estimation of proximity and rotational mobility of receptors by using only a single cell sample in steady state conditions. The fact that the extent of rotational motion can be estimated besides FRET may gain special importance, because it may offer the feasibility for the estimation of orientation factor (κ^2) and consequently the distance from the measured homo-FRET efficiencies [22–24].

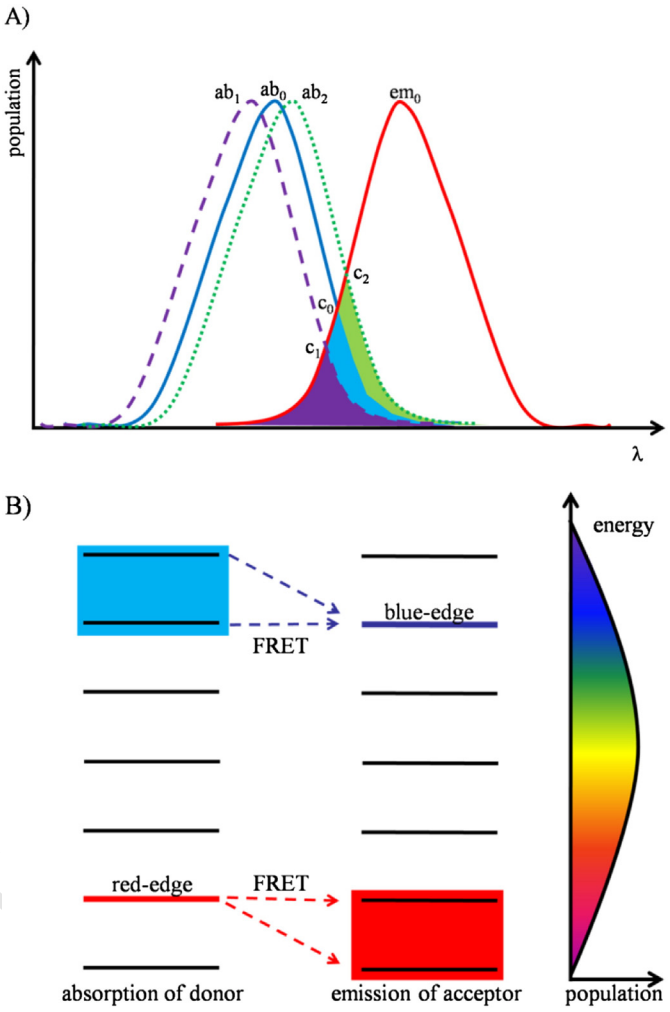

Fig. 1. Cartoons explaining how inhomogeneous broadening induces directed FRET migration. Panel A: Illustration of the mechanism for FRET directionality. A dye with absorption spectrum ab_0 (blue) and emission spectrum em_0 (red) transfers energy with larger probability to dye with absorption spectrum at longer wavelengths like ab_2 (green) than to those with absorption spectrum at shorter wavelength like ab_1 (violet), due to the larger spectral overlap for FRET. While the overlap between em_0 and ab_2 is larger than the overlap between em_0 and ab_0 with the green portion (with apex c_2), the overlap between em_0 and ab_1 (violet area with apex c_1) is smaller than the overlap between em_0 and ab_0 with the blue portion (with apex c_0). This leads to net energy migration in the direction of decreasing energy levels, like water flows from the hills towards the walleys [16,20]. The emission spectrum em_0 is shifted to the right as compared to ab_0 due to the Stokes-shift. The emission spectra belonging to absorption spectra ab_1 and ab_2 are not indicated. Panel B: Energy level diagrams for the potential FRET donors (left) and acceptors (right) from an ensemble of a given type of dye. The different solvent microenvironments introduce energetic heterogeneity in the dye population by splitting up a single energy level (the middle one at yellow) into a set of sublevels (3 new levels above and under the middle one). The rainbow-colored contour to the right of the energy ladders represents the population distribution on the energy levels. Directionality in FRET is introduced by the fact that – due to the Stokes-shift, which is not indicated for easiness – overlap integral is larger for those acceptor levels which lie under the donor level from which FRET starts out [16,20] (see also Panel A). A consequence is that FRET predominantly happens towards acceptors of energy levels lower than for the donor leading to a FRET-correlated red shift of the emitted light. For the same reason, the potential acceptor subpopulations for FRET directed from the lower lying donor levels, i.e., at the red-edge, are severely restricted, and manifested in the loss of FRET. Similarly, when selectively monitoring the blue-emitting dyes, i.e., at the emission blue-edge, these species can be potential FRET acceptors only for the very few donors lying upwards in energy, implying a corresponding loss of FRET. Our method is based on an optimal choice of a FRET sensitive (absorption at the main-band and detection at the red) and an insensitive (absorption at the red-edge and detection close to the blue-edge) anisotropy channel. Determination of an absolute homo-FRET efficiency may be possible by the elimination of the dependence on rotation by ratioing the two anisotropies, whenever they have equal sensitivities for the rotation.

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