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Assessing FRET Using Spectral Techniques

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THE manuscript "Assessing FRET using spectral techniques" by Leavesley et al. (page XXX in this issue) addresses Förster resonance energy transfer (FRET) between organic molecules due to an interaction of optical transition dipole moments. Although occurring in nature for more than a billion of years, a theoretical description of this mechanism has only been given in 1948 (1). Since that time energy transfer spectroscopy has been used to probe molecular distances and mechanisms in the nanometre range (2–4), profiting from the fact that the energy transfer rate depends on the sixth power of molecular distance ($k_{ET} \sim r^{-6}$) and is, therefore, a very sensitive parameter. FRET experiments gained considerable importance, when in the 1990s green fluorescent protein [GFP, naturally occurring in the jellyfish *Aequorea Victoria*, (5)] and its mutants could be cloned and fused with almost any protein of a cell. So, nonradiative energy transfer from cyan fluorescent protein (CFP) to yellow fluorescent protein (YFP) or from GFP to red fluorescent protein was used to probe changes of molecular conformation, for example, upon binding of calcium (6), or interactions between adjacent molecules, for example, protein−protein interactions playing an important role in regulation of apoptosis (7) or in pathogenesis of M. Alzheimer and further neurodegenerative diseases (8,9).

Förster energy transfer commonly arises, if a so-called donor molecule is excited by light and transfers its excitation energy to an adjacent acceptor molecule via dipole–dipole interaction. The latter commonly fluoresces at longer wavelength. Generally, two approaches are used to quantify this process:

1. The ratio of acceptor/donor fluorescence quantum flux is determined upon stationary optical excitation of the donor. This method has several restrictions: (i) the fluorescence quantum yields of donor and acceptor should be well known, (ii) simultaneous direct excitation of the acceptor should be avoided or otherwise taken into account quantitatively, and (iii) donor and acceptor fluorescence should be distinguished according to their emission spectra, which often requires complex deconvolution algorithms.
2. Energy transfer can also be deduced from fluorescence lifetime of the donor molecules after short (commonly picosecond) pulse excitation. If the rate of deactivation k of an excited molecular state is described by the sum of radiative (k_r) and nonradiative (k_{nr}) transitions as well as transitions via intermolecular energy transfer (k_{ET}), and if the lifetime of this excited state (fluorescence lifetime) τ is equal to $1/k$, the energy transfer rate can be determined as

$$k_{ET} = 1/\tau - 1/\tau_0 \quad (1)$$

with τ corresponding to the fluorescence lifetime of the donor in presence and τ_0 in absence of energy transfer. For calculation of the energy transfer rate k_{ET} neither the fluorescence quantum yields of donor and acceptor molecules have to be determined, nor are any complex deconvolution procedures necessary, if background fluorescence (e.g., autofluorescence) can be sufficiently suppressed. Measurement of donor fluorescence without spectral overlap by the acceptor, however, is needed, and the fluorescence lifetime τ_0 has to be determined from a reference experiment where the acceptor is either missing or photobleached [(10), with the risk of pronounced cell damage]. In addition, light sources of picosecond duration as well as detection systems with subnanosecond time resolution should be available.

Due to these experimental restrictions (and pronounced costs) of time-resolving FRET experiments, spectral techniques and algorithms upon stationary optical excitation still play an important role, as outlined in the manuscript by Leavesley et al. (page XXX in this issue). Here, the authors used various techniques and algorithms based on up to three filter sets (e.g., for measurement of FRET efficiency at variable

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donor concentration) as well as spectral unmixing. Using a probe, where CFP and YFP are linked by a cAMP binding protein, they measured FRET efficiency in cultivated cells as a function of cAMP concentration. Although a two-filter set approach was already able to describe FRET efficiency with rather low coefficients of variation, spectrally resolved experiments provided improved results. The authors concluded that hyperspectral confocal microscopy in combination with linear unmixing, cell segmentation, and quantitative image analysis was appropriate for FRET experiments in single cells. I would like to share this opinion, emphasizing that in an ideal case spectral and time-resolving FRET experiments might be combined. This holds in particular, if acceptor fluorescence is low (8), or if donor and acceptor fluorescence are spectrally indiscernible [Homo FRET, (11)].

During the last years resolution in optical microscopy has been improved considerably. Starting from a value around 200 nm, as resulting from Abbé's diffraction theory, values of 10−30 nm were meanwhile obtained by super-localization microscopy based on single molecule detection (12,13) or by stimulated emission depletion microscopy [STED, (14)]. Resolution in FRET experiments still goes below these values and permits to measure molecular distances of 5 nm and less. In addition, high irradiance as needed for STED (and to some extent also for single molecule detection) and risking severe cell damage can be avoided in FRET experiments. Furthermore, FRET is not restricted to microscopy and not limited to adherent samples, but can be combined with any other method, for example, flow cytometry (15).

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