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Optical analysis of cellular oxygen sensing

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ABSTRACT

Molecular imaging of the assembly of hypoxia inducible factor (HIF) complexes in living cells may lead to a deeper understanding of cellular oxygen sensing. Sophisticated live cell imaging has extended the toolbox to study the molecular response to changes in oxygen supply. In this respect fluorescence resonance energy transfer (FRET) as a technique to investigate protein-protein interaction in the nanoscale range gets increasing interest. Herein, we review FRET studies related to hypoxia research, emphasizing on recent progress, but also demonstrating how FRET studies are complementary or potentially superior to conventional biochemical as well as histochemical techniques. Technical advances in the application of FRET in living cells will overcome restrictions to end-point analysis on the population rather than single cell level and will thereby provide progress in understanding the cellular hypoxic response by HIF.

1. Introduction

During the last decades, advanced microscopy imaging methods have become increasingly popular to study molecular and cellular mechanisms. Mainly technical limitations, e.g. the precise control in oxygen supply in the microscope setup or in fluorescence readers, hampered live cell imaging under hypoxia. An increasing number of publications within the last 5 years demonstrate the clear advantages of live cell imaging approaches like fluorescence resonance energy transfer (FRET) and fluorescence recovery after photobleaching (FRAP) to complete the toolbox in hypoxia related research. Quantitative methods to address protein-protein interactions on the nanoscale within living cells, like fluorescence lifetime imaging microscopy (FLIM), became available. Hence, quantitative investigation of cell signalling in correlation with high spatio-temporal resolution and cell fate will promote progress in basic research investigating oxygen sensing mechanisms.

1.1. Fluorescence resonance energy transfer (FRET)

Different approaches to investigate protein-protein interaction using fluorescence resonance energy transfer (FRET) have evolved and have been reported since the first description by Förster in 1948 [1]. FRET is the non-radiative energy transfer from an excited donor fluorophore to an acceptor fluorophore in close proximity (< 10 nm). The FRET efficiency (E) depends on the Förster radius of the FRET pair molecules R_0 and the distance r (1) [2]:

$$E = \frac{{R_0}^6}{{R_0}^6 + r^6} \text{ with } R_0(\text{Å}) = 0.\ 221[\kappa^2 n^{-4} Q_D J(\lambda)]^{1/6} \quad (1)$$

where κ^2 is the dipole-dipole orientation factor of the FRET fluorophore pair, n is the medium refractive index, Q_D is the quantum yield of the donor alone and J (λ) is the spectral overlap integral of the donor emission and acceptor absorption with the wavelength expressed in nm. For free rotating donor and acceptor molecules κ^2 is 2/3. Parallel dipole orientation would allow maximal FRET, while perpendicular orientation prevents FRET [2].

All FRET measurements share common prerequisites. A donor and an acceptor fluorophore, with a sufficient overlap integral in the donor emission and acceptor excitation spectra allow radiation-free energy transfer from the excited donor to an acceptor molecule. The energy transfer only occurs within a distance less than approximately 10 nm.

Abbreviations: HIF, hypoxia inducible factor; FRET, fluorescence resonance energy transfer; FRAP, fluorescence recovery after photobleaching; FLIM, fluorescence lifetime imaging microscopy; apFRET, acceptor-photobleaching FRET; YFP, yellow fluorescent protein; seFRET, sensitized-emission FRET; TCSPC, time correlated single photon counting; pVHL, van Hippel-Lindau protein; (E)CFP, (enhanced) cyan fluorescence protein; DMOG, dimethyloxalylglycine; TADs, transactivation domains; CH domains, cysteine/histidine-rich regions; CBP, CREB-binding protein; FIH, factor inhibiting HIF–1; PHD, prolyl hydroxylase domain; IPAS, Inhibitory PAS domain protein; BAX, BCL2-associated protein X

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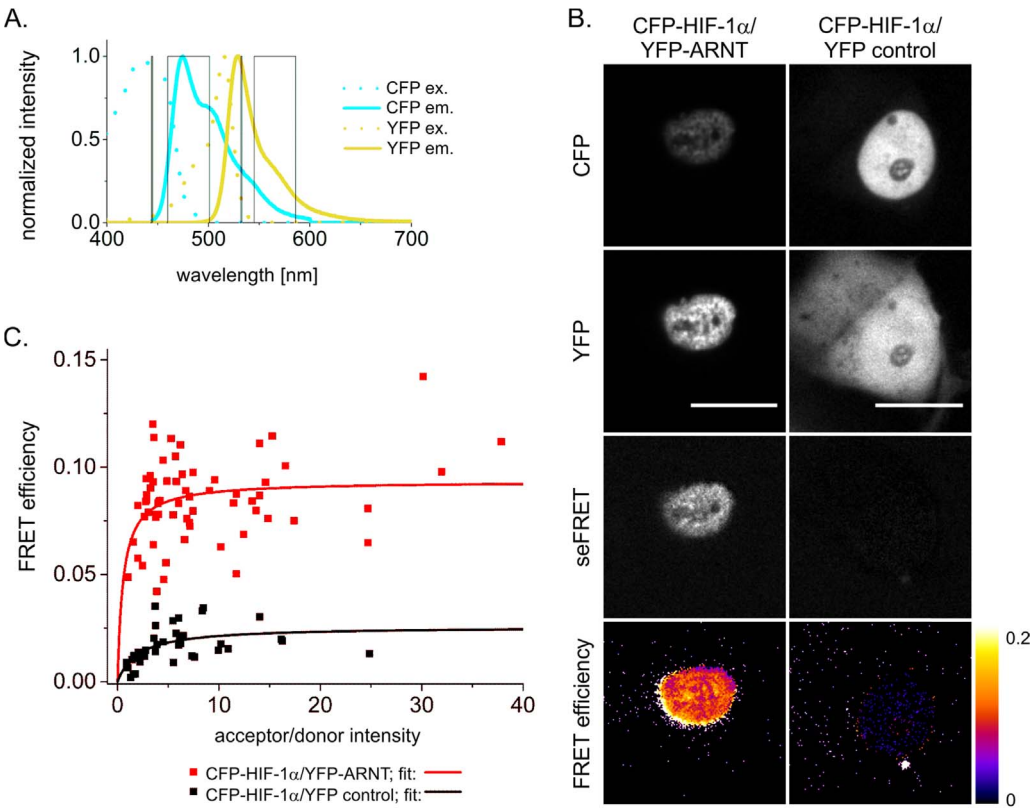

Fig. 1. HIF-1α/HIF-1β heterodimerization proven by sensitized-emission FRET. A. Peak-normalized spectra of CFP (donor) and YFP (acceptor) fluorophore excitation and emission. Vertical lines indicate laser excitation at 444 nm (donor) and 532 nm (acceptor). Respective emission bandpass filter sets are indicated by boxes (460–500 nm (donor); 545–585 nm (acceptor)). B. Representative intensity and bleed trough corrected seFRET images of live U2OS cells transfected with CFP-HIF-1α and YFP-HIF-1β (left) or CFP-HIF-1α and soluble YFP control (right). Scale bars 15 μm. C. FRET efficiency of individual living U2OS cells transfected with CFP-HIF-1α and YFP-HIF-1β (N=64) or CFP-HIF-1α and soluble YFP control (N=40) as negative control. FRET efficiency plateaus at 0.093 +/− 0.005 for CFP-HIF-1α and YFP-HIF-1β heterodimerization, but only 0.026 +/− 0.004 for the negative control (sigmoidal fit).

Thus, FRET signals derived from fluorophores attached to interacting proteins strongly correlate with physical interaction of these proteins. In order to avoid false-positive FRET signals, donor as well as acceptor fluorophores should not oligomerize. For detailed information on fluorescent protein FRET pairs and their use in diverse FRET approaches we recommend a recent comprehensive review [3]. A multitude of original articles and detailed reviews on FRET techniques and analysis have been published, covering the most recent developments [4–7]. Simply spoken, FRET efficiency depends on the proximity of the fluorophore-labelled proteins in questions. Within the above mentioned limits FRET may be used to calculate distances between proteins in living cells in the nanometer range such as in the HIF complex. Herein, we focus on the advantages and usability of different approaches to measure FRET in cellular context, specifically in live cell microscopy under hypoxia.

1.2. Acceptor-photobleaching FRET

An easy method to assess FRET is acceptor-photobleaching (apFRET) [8]. Since the measurement can be performed with a conventional widefield fluorescence microscope using three sets of filters it is rather cheap and accessible. After donor excitation fluorescence from the donor and the acceptor is recorded. Then light from a laser of an appropriate wavelength is used to bleach the acceptor fluorophore. Due to photobleaching of the acceptor, energy transfer from donor to acceptor is impeded and donor fluorescence emission intensity rises. FRET efficiency is thus calculated using pre- and post-bleach images of donor and acceptor fluorescence intensities of the sample. Acceptor-photobleaching FRET allows a high spatial resolution of the interacting fraction, while repeated measurement of FRET within one sample is not possible due to the irreversible nature of photobleaching. The advantage in the context of oxygen sensitive measurements is the option to chemically fixate the sample under low oxygen and investigate them afterwards using a standard widefield or laser-scanning microscope without the need of a hypoxia chamber attached to the microscope. When yellow fluorescent protein (YFP) derivatives are used as acceptors, the experiment should be controlled for photoconversion [9]. Otherwise, acceptor-bleaching by high doses of blue light could induce photoconversion of the acceptor to a fluorophore with similar spectral properties as the donor and therefore lead to artificially high (false-positive) FRET efficiency.

1.3. Sensitized-emission FRET

Sensitized-emission FRET (seFRET) describes the direct detection of acceptor emission upon donor excitation. It offers the option to measure comparably fast processes with good spatial resolution on subcellular level without the need of specialized microscopy equipment [10]. It is semi-quantitative and very useful to investigate whether proteins in living cells interact or not. It is even possible to achieve relative changes upon system perturbation, such as the addition of an inhibitor. Sensitized emission measurements allow a fast acquisition of multiple individual living cells in different environments and correlation with relative ratios of donor and acceptor intensities. On the other hand, it requires a very careful and repetitive set of controls, adjustment of the microscope and the analysis software due to inter-individual differences between days of measurement [11]. A lot of system calibration including donor- and acceptor-only samples in