Chapter 15

Monitoring of Cellular Responses to Hypoxia

Christoph Wotzlaw and Joachim Fandrey

Abstract

Oxygen is essential for survival of aerobic organisms. Sensing changes in the environmental oxygen concentration and appropriate adaptation to such changes are essential for organisms to survive. Hypoxia inducible factor 1 (HIF-1) is the key transcription factor in controlling the expression of oxygen-dependent genes required for this adaptation. HIF-1 is a heterodimer of an oxygen dependent α-subunit and constitutive β-subunit. Abundance and activity of HIF-1 is controlled by post-translational hydroxylation. Microscopic analysis of the assembly and activation process of HIF-1 has become an important tool to better understand HIF-1 regulation. Confocal laser microscopy provides exact images of HIF-1α that is translocated into the nucleus under hypoxia and its disappearance upon reoxygenation. To exactly follow the protein–protein interaction during the assembly process of HIF-1, both subunits were labeled by fusing them to fluorescent proteins. Fluorescence resonance energy transfer (FRET) was used to determine the interaction of both subunits in living cells by confocal microscopy.

Key words: Oxygen sensing, hypoxia inducible factor 1, HIF-1, protein–protein interactions, fluorescence resonance energy transfer (FRET), confocal microscopy.

1. Introduction

When oxygen supply to the tissue is reduced during ischemia or impaired respiration, a state of hypoxia develops. Organisms have to adapt to hypoxia to avoid severe damage or even death of respective tissues or the whole organism. Common to all countermeasures to ameliorate hypoxia is the ability of cells to rapidly detect changes in oxygen concentration (1). The adaptive response that is then initiated may include changes in oxygen capacity of the blood, improved blood circulation and ventilation or a switch to anaerobic metabolism. These responses depend on

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acute changes of different ion channel conductivities and subsequently cell membrane potential but also on long term changes in expression of a considerable number of genes. Hypoxia inducible factor 1 (HIF-1) is the transcriptional factor that coordinates central response of cells and organs to hypoxia (2). HIF-1 is composed of an oxygen labile α-subunit (HIF-α) and a constitutively expressed nuclear β-subunit (HIF-1β). Regulation of HIF-1α abundance is achieved through post-translational hydroxylation of specific proline residues within this subunit by oxygen-dependent prolyl hydroxylases, which thus act as bona fide cellular oxygen sensors. While hydroxylation initiates proteasomal degradation of HIF-1α under normal oxygen concentrations, reduced activity of oxygen sensor hydroxylases under hypoxia allows HIF-1α to evade destruction and form the HIF-1 dimer with its β-subunit (3). Similarly, oxygen-dependent hydroxylation of an asparagine residue within the HIF-1α protein controls transcriptional activity of the HIF-1 complex (3). One of the many target genes activated by HIF-1 under hypoxia is erythropoietin, the key regulator of red blood cell production (4). Erythropoietin is the paradigm of a hypoxia-induced gene which had helped to decipher HIF-1 activation and cellular oxygen sensing. However, it has not been resolved yet how stabilized HIF-1α interacts with its partners, both HIF-1β to form the HIF-1 dimer and also coactivators which are required for transcriptional activity. Recently, we have introduced a fluorescence energy transfer technique to visualize for the first time the interaction between the two subunits of HIF-1 and monitor the assembly of HIF-1 complex in living cells (5). In addition, we have developed and optimized scanning procedures, specialized software and evaluation processes that can also be applied to the studies of protein–protein interactions in living cells and some other models (6–8). In this paper we describe confocal imaging of HIF-1α and its nuclear distribution. In a specialized chamber which allows to study living cells under well-defined hypoxia through the microscope we follow the dimerization of HIF-1α and HIF-1β.

2. Materials

2.1. Cell Culture

1. Dulbecco's modified Eagle's medium (DMEM with 4.5 g/l glucose, L-glutamine, and pyruvate; all from Invitrogen GmbH, Karlsruhe, Germany) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml; Invitrogen GmbH, Karlsruhe, Germany) and 10% fetal bovine serum (FBS; Biocompare, South San Francisco, CA).

2. DMEM without FBS for plasmid transfection.
3. 10× stock of phosphate buffered saline (PBS), which contains 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4 (adjusted with HCl), autoclaved. PBS is prepared by 1:10 dilution of stock with distilled water.
4. Solution of trypsin (0.25%) and ethylenediaminetetraacetic acid (EDTA) (1 mM).
5. Cell culture flask (75 cm^2 bottom area) (Greiner Bio-One GmbH, Solingen, Germany).
6. Freely available software "ImageJ" from the National Institutes of Health, USA (http://rsbweb.nih.gov/ij).

2.2. Immunofluorescence of Hypoxia Inducible Factor 1α

1. 24-well polystyrene plates with 1.9 cm^2 growth area per well (Greiner Bio-One GmbH, Solingen, Germany).
2. Glass cover slips with a diameter of 1.2 cm (Glaswarenfabrik Karl Hecht KG "Assistent," Sondheim/Rhön, Germany).
3. 10× stock of PBS (*see* **Section 2.1**, Step 3). PBS is prepared by 1:10 dilution of stock with distilled water.
4. Fixation solution: 1:1 (v/v) methanol/acetone, stored at –20°C.
5. Permeabilization solution: 0.5% (v/v) Triton X-100 (Invitrogen GmbH, Karlsruhe, Germany) in PBS.
6. Mouse monoclonal anti-HIF-1α antibody (Transduction Lab., Lexington, KY).
7. Secondary goat anti-mouse IgG conjugated to Alexa Fluor 568 (Invitrogen GmbH, Karlsruhe, Germany). Caution: the conjugate is light sensitive.
8. Nuclear stain Hoechst33342 (Invitrogen GmbH, Karlsruhe, Germany), 5 μM solution in water. Caution: the fluorophore is light sensitive.
9. Antibody dilution buffer: 3% (w/v) bovine serum albumin (BSA) in PBS.
10. Anti-fading and mounting reagent Prolong Gold (Invitrogen GmbH, Karlsruhe, Germany).
11. Standard inverted fluorescence confocal microscope, one or two photon excitation.
12. Standard incubator (Heraeus) for cell culture, set at 37°C, 5% CO_2, and 100% humidity.
13. Hypoxia incubator (Heraeus) with adjustable oxygen concentration, down to 1% O_2.

2.3. Microscopy Study of HIF-1α Under Defined Gas Composition and Interaction Analysis Between HIF-1 Subunits Using Sensitized FRET Technique

1. Fugene6 transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany).
2. Fusion proteins: HIF-1α fused to enhanced cyan fluorescent protein (ECFP) (Clontech GmbH, Germany) and HIF-1α fused to enhanced yellow fluorescent protein (EYFP) (Clontech GmbH, Germany) – ECFP-HIF-1α and EYFP-HIF-1β, respectively.
3. Glass-bottom cell culture dishes, 3.5 cm in diameter (WillcoWells BV, Amsterdam, the Netherlands). Physical characteristics of central glass insert allow all standard formats of microscopy including UV light excitation.
4. Chamber for cell culture dishes (Luigs & Neumann, Ratingen, Germany) for observation of living cells on microscope stage under the conditions of controlled temperature, humidity and gas composition, e.g., O_2 and CO_2 (5). Gas concentrations are individually adjustable by a gas mixing device (Newport Spectra-Physics GmbH, Darmstadt, Germany) connected to the chamber.
5. For microscopy of HIF-1α under different oxygenation conditions, a standard inverted confocal microscope with objective lenses plan apochromat 40× (Nikon GmbH, Düsseldorf, Germany) is used.
6. For sensitized FRET analysis of HIF-1α-HIF-1β interaction, a confocal microscope is used with one excitation laser line between wavelengths of 405 and 444 nm for ECFP (termed "donor" for FRET measurements) and a second laser between wavelengths of 514 nm and 532 nm for EYFP (termed "acceptor" for FRET) excitation.
7. Recommended filters FRET: Two band pass emission filters – 480/30 and 545/40 nm (AHF AG, Tübingen, Germany).

3. Methods

3.1. Cell Culture

Procedures described below are performed on human osteosarcoma cell line U2OS. Other cell lines available from the American Type Culture Collection (ATCC, Manassas, VA) such as hepatoma (HepG2) or hepatocellular carcinoma cells (Hep3B), and human embryonic kidney 293 cells containing the SV40 large T-antigen (293T) can also be used.

In samples with high cell density hypoxia at the bottom of cell culture flask may develop during experiment if oxygen supply to the cells is limited by diffusion (9). Therefore, for flasks with 75 cm^2 bottom area we recommend a moderate density of cells

not exceeding 80% confluency. For immunofluorescence experiments cells are grown on glass cover slips in 24-well plates. Cover slips are sterilized by dipping them in 95% ethanol.

Cells in a flask are detached using the addition of 2 ml trypsin/EDTA. Two minutes later, trypsin is inactivated with 8 ml DMEM-FBS, and 30 μl of the suspension are transferred with a pipette into the wells containing 500 μl DMEM-FBS. The plate is steadily shaken to obtain a uniform cell spreading.

For experiments, 130 μl of cell suspension are taken and pipetted into sterile 3.5-cm dishes containing 1 ml DMEM–FBS. The dish is steadily shaken to obtain uniform cell spreading. The plate or dishes with cells are then put in CO_2 incubator for 3–6 h to allow the cells to attach. Immunofluorescence staining can be done 24–48 h later.

3.2. Immunofluorescence of HIF-1α

1. Seed cells and grow them on sterile cover slips in 24-well plates as described in **Section 3.1** (*see* **Note 1**).
2. Expose cells for 4 h to four different conditions: (i) ambient O_2 concentration (normoxia); (ii) low O_2 concentration, 1% O_2 (hypoxia); (iii) 100 μM of $CoCl_2$ in DMEM-FBS at normoxia – a common mimetic of hypoxia (4); (iv) hypoxia and subsequent reoxygenation (*see* **Note 2**).
3. After incubation, take all plates out and quickly rinse twice with ice cold PBS, except for plates subject to reoxygenation. These plates are placed into normoxia incubator for 5 min and then rinsed with ice cold PBS twice.
4. Add ice cold methanol/acetone (1:1) and incubate for 5 min to fix the cells. Then discard the solution, wash the cells briefly with PBS, and discard PBS.
5. Permeabilize cells by incubating the samples in Triton X-100/PBS for 5 min at room temperature, then wash with PBS again. For some cells such as U2OS this step is unnecessary.
6. Block samples for 15 min in antibody dilution buffer (3% (w/v) bovine serum albumin in PBS); discard buffer afterwards.
7. Incubate samples at room temperature in a volume of 200 μl with mouse monoclonal anti-HIF-1α antibody diluted 1:50 in buffer for 60 min.
8. Wash samples three times for 5 min with PBS.
9. Add secondary antibody diluted 1:400 in antibody dilution buffer (volume: 200 μl) and incubate for 30–60 min at room temperature (*see* **Note 3**). Discard secondary antibody.

10. For nuclear staining (optional), add 200 µl of 5 µM Hoechst33342 solution and incubate for 5–10 min at room temperature.
11. Wash samples three times for 5 min with PBS and dry.
12. Add 2.5 µl of anti-fading and mounting medium to samples on cover slips which are then put on microscopy slide. Dry slides in the dark at room temperature for at least 3 h before measurements.
13. View slides under a wide-field microscope with selected excitation and emission wavelengths or on a confocal microscope with laser light excitation. Alexa Fluor568 is excitable at wavelengths 500–600 nm and emits fluorescence at 580–700 nm. Hoechst33342 is excitable between 300 and 400 nm and emits in the range of 400–600 nm. Due to the distinct spectra of the fluorophores, no crosstalk perturbs images when localizing HIF-1α relative to the nucleus (*see* **Fig. 15.1**).

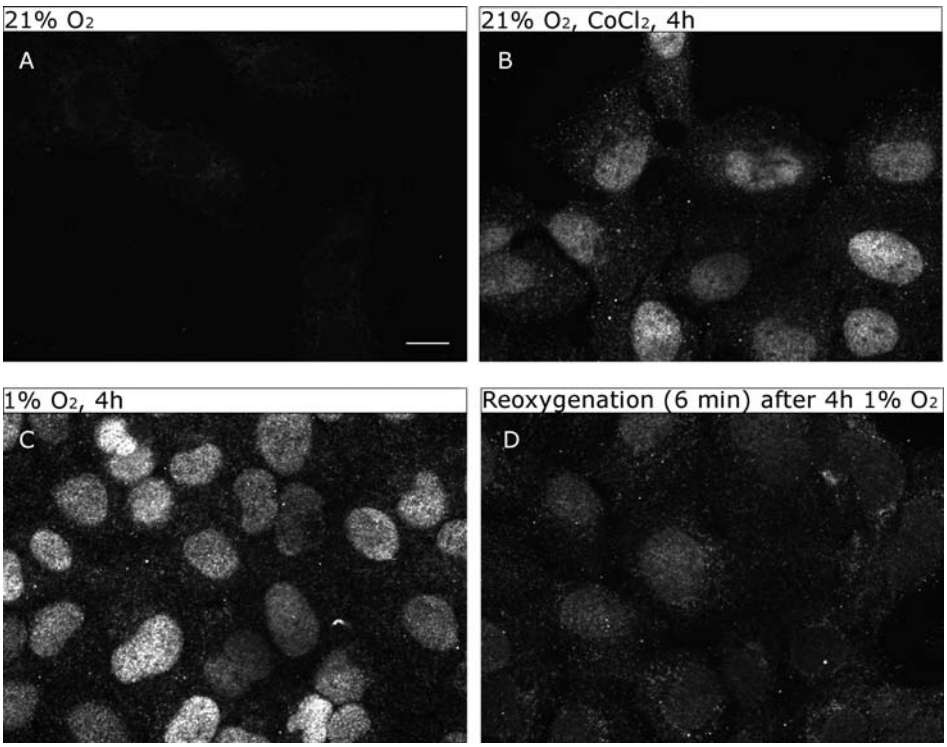

Fig. 15.1. Endogenous HIF-1α detected by immunohistochemistry. **(a)** Only weak signals from small amounts of HIF-1α protein are detected under normoxic conditions (21% O_2) due to oxygen dependent degradation of HIF-1α. **(b)** Nuclear accumulation of HIF-1α after treatment with hypoxia mimetic $CoCl_2$, which inhibits degradation of HIF-1α. **(c)** Hypoxia induced accumulation of HIF-1α in the nucleus due to reduced degradation. Oxygen serves as substrate for prolyl hydroxylases, which act as oxygen sensor through initiation of normoxic degradation of HIF-1α. **(d)** Rapid decrease of cellular HIF-1α protein upon reoxygenation. The return of oxygen after a hypoxic period enhances prolyl hydroxylase activity and initiates the fast degradation of HIF-1α within a few minutes.

3.3. Microscopic Study of HIF-1α Under Defined Gas Composition

1. Seed and grow cells onto sterile 3.5 cm dishes as described in **Section 3.1**.
2. Mix in eppendorf tube 4.5 μl of Fugene6 with 150 μl DMEM *without* serum. After 5 min, add 1.5 μg plasmid DNA encoding ECFP-HIF-1α, gently shake, and incubate for 30–40 min at room temperature.
3. Add this solution to the dish with cells, gently shake, and incubate for 6 h to allow transfection of cells with HIF-1α fusion protein.
4. Replace medium with fresh DMEM-FBS (*see* **Note 4**). Transfection is now completed.
5. Incubate cells for further 24–48 h. This allows synthesis of fusion proteins from plasmids in sufficient quantities so that fluorescence signal is bright enough for microscopic analysis.
6. Place one dish on microscope stage into the chamber adjusted to desired temperature and gas composition. Equilibrate cells for at least 15 min to these conditions.
7. Scan a stack of 6–8 fluorescent images of the middle part of the cell which shows high fluorescence intensity. The distance between sections/planes in *z*-direction should be kept between 500 and 700 nm. This allows evaluation of experimental data even if the cell moves along the optical axis during scanning (*see* **Note 5**).
8. Switch O_2 concentration to 1% and after 2 h take a new stack of 3D image.
9. Open the first 3D stack in image analysis software (*see* **Note 6**). Select the image with highest HIF-1α fluorescence and, after subtracting background value, calculate mean fluorescence. Process the second 3D stack similarly. Present results as a diagram.

3.4. Analysis of Interactions Between HIF-1 Subunits Using Sensitized FRET Technique

According to the theory of fluorescence resonance energy transfer (FRET), energy from the donor molecule is transferred nonradiatively to the acceptor if the two molecules come closer than a critical distance value. For the majority of fluorophore pairs this value is near 10 nm. In this case, when exciting the donor, fluorescence of the acceptor can be recorded (*see* **Fig. 15.2**).

Microscopic study of interaction between proteins requires initial calibration procedures, in which the main characteristics of the imaging system and fluorophores and "spectral bleedthrough" values are determined. Protein–protein interaction analysis under the microscope requires labeling of the two proteins with different fluorophores. In the case of FRET, excitation of the donor molecule will initiate acceptor fluorescence if the distance between the two protein–fluorophore molecules is less than

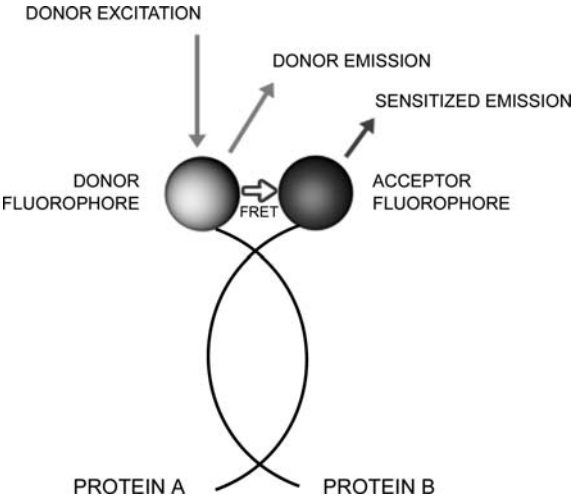

Fig. 15.2. Schematic description of fluorescence resonance energy transfer (FRET) to detect protein–protein interaction in involved living cells. Interaction of proteins requires close neighborhood of the molecules. Proteins can be labeled in living cells by generating fusion proteins containing a fluorophore and target protein. If the two fluorophore-labeled proteins come closer than 10 nm to each other due to their interaction, energy from the donor fluorophore which has been excited by laser light can be non-radiatively transferred to the acceptor fluorophore causing sensitized emission of light at the emission wavelength of the acceptor. The closer the two proteins, the greater the proportion of energy transferred to the acceptor and the smaller the proportion of residual donor emission.

10 nm. Since common fluorophores show broad excitation and emission bands, the acceptor can also be excited directly by donor excitation light, and donor emission may be collected in the acceptor emission channel. This effect, called bleed-through from one fluorophore channel to the other, has to be determined individually for each microscopy system and fluorophore combination. Bleed-through values are needed for correct quantification of non-radiative energy transfer from donor to acceptor.

1. Seed and grow cells on sterile 3.5 cm dishes as described in **Section 3.1**.
2. Perform transfection with following fusion protein constructs: (a) pECFP-HIF-1α only, (b) pEYFP-HIF-1β only, (c) pECFP-HIF-1α and pEYFP-HIF-1β, (d) pECFP and pEYFP. Add to each dish 4.5 μl of Fugene6 mixed in eppendorf tube with 150 μl of DMEM *without* FBS. After 5 min add 1.5 μg plasmid DNA of each construct, gently shake the tube with the transfection solution and incubate for 30–40 min at room temperature. Then add the transfection solution to the cell culture dish and gently shake the dish. Incubate cells for 6 h, then change medium for fresh DMEM-FBS.

3. Grow cells for further 24–48 h after transfection to allow synthesis of fusion proteins from plasmids and sufficiently high fluorescent signals for microscopic analysis.
4. Place dish into microscope chamber set to 21% O_2, 79% N_2, and 5% CO_2 (normoxia) and 37°C. Adapt cells to these conditions for at least 15 min before starting microscopic analysis.
5. Perform calibration process for each protein–protein interaction analysis as follows (*see* **Note 7**). Determine the degree by which donor fluorescence is detected in the acceptor channel (relative to donor fluorescence in the donor channel) using appropriate excitation and emission filters for the donor. Image five cells transfected only with ECFP-HIF-1α donor by exciting them with a laser line between 405 and 444 nm (optimal for ECFP) and collecting donor fluorescence with (i) the acceptor filter (band pass emission filter: 545/40 nm, AHF AG, Tübingen, Germany) to produce an image called *BTfd*; (ii) with the donor filter (band pass emission filter: 480/30 nm, AHF AG, Tübingen, Germany) to produce an image called *BTd*).
6. Determine the degree by which acceptor fluorescence is detected in the acceptor channel under donor excitation (relative to acceptor fluorescence in the acceptor channel). Image five cells transfected with EYFP-HIF-1β acceptor only as follows: (i) excite them with the donor laser and collect fluorescence with the acceptor filter to produce an image called *BTfa*; (ii) excite with a laser line between 514 and 532 nm (optimum for EYFP) and collect fluorescence with the acceptor filter to produce an image called *BTa*. This measurement takes into account that EYFP molecules are excitable to a certain degree by the laser used for donor excitation.
7. Determine background by scanning an area outside fluorescent cells. Background values are subtracted from every image.
8. Calculate calibration parameters:

$$BTfd/BTd = BTD\,(\text{spectral bleed-through of the donor}) \quad [1]$$

$$BTfa/BTa = BTA\,(\text{spectral bleed-through of the acceptor}) \quad [2]$$

   Mean BTD and BTA values in cells are calculated.
9. For the analysis of protein–protein interaction, image at least 20 cells transfected with both fluorescent donor ECFP-HIF-1α and acceptor EYFP-HIF-1β as follows: (i) excite with the donor laser and collect fluorescence with

the donor filter, to produce an image called *donor image*, *D*; (ii) excite with the donor laser and collect fluorescence with the acceptor filter, to produce an image called *FRET image*, *F*; (iii) excite with the acceptor laser and collect fluorescence with the acceptor filter to produce an image called *acceptor image*, *A*.

10. Determine background values by scanning areas outside fluorescent cells and subtract them from every image. Then calculate mean fluorescent signals for each cell.
11. Evaluate images of co-transfected cells. In the case of FRET from ECFP donor to EYFP acceptor due to close proximity of the two HIF-1 subunits ($< \sim 10$ nm), the percentage of transferred energy relative to the energy absorbed by the donor determines *FRET efficiency*. It can be calculated for every pixel of a FRET image using the following formula:

$$\text{FRET efficiency (\%)} = [1 - [D/(D + F - D \times BTD - A \times BTA))] * 100, \quad [3]$$

where *D* is donor fluorescence in the donor channel under donor laser excitation; *F* is fluorescence in the acceptor channel under donor laser excitation; *A* is acceptor fluorescence in the acceptor channel under acceptor laser excitation; *BTD* and *BTA* are spectral bleed-through values of the donor and acceptor, respectively; and $(F–D \times BTD–A \times BTA)$ is the energy transferred from ECFP to EYFP detected as EYFP fluorescence (s*ee* **Note 8**).

12. Calculate mean FRET efficiency values for fluorescent nuclei of co-transfected cells. FRET efficiency is also influenced by the number of acceptor molecules surrounding a donor molecule (8). To take this fact into account the system specific ratio of acceptor to donor molecules is determined as the ratio of the fluorescence from acceptor molecules to that of the donor molecules; FRET efficiencies are presented as values over this ratio. **Figure 15.3a** shows the example of HIF-1α/β FRET in U2OS cells, where the image represents typical distribution of fluorescent signals within the cell and the graph represents regression curve of mean FRET values from ~20 cells with different acceptor/donor molecule ratios.
13. Account for *random FRET*, which is caused by random collisions of fluorophores during the time of imaging. In addition, some fluorophores derived from *green fluorescent protein* (GFP) such as ECFP and EYFP tend to form dimers causing false-positive signals (*see* **Note 9**). Therefore, control measurements need to be done with cells cotransfected

with pECFP and pEYFP fluorophore plasmids, rather than fusion proteins of HIF-1 subunits. Experimental procedure is the same as in **Section 3.4**. Mean FRET efficiency values of cotransfected cells are calculated and presented as dependence of the ratio of acceptor and donor fluorescence (*see* **Fig. 15.3**).

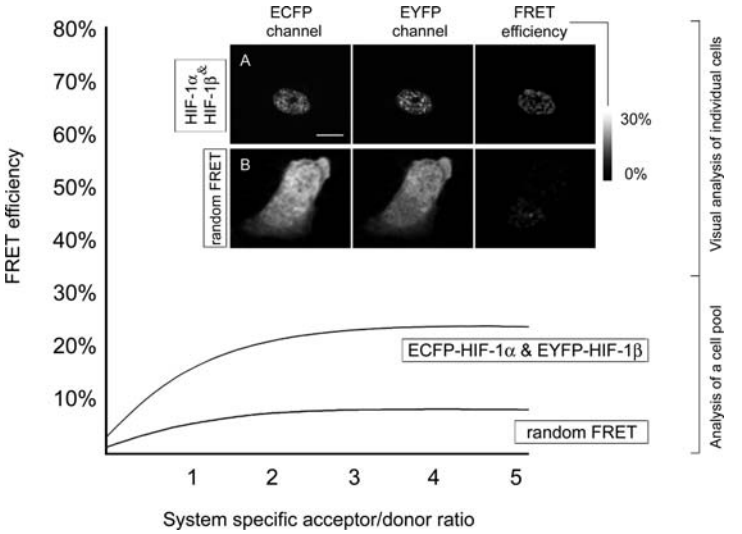

Fig. 15.3. Microscopic evaluation of FRET between HIF-1α and HIF-1β. FRET efficiency is a function of the ratio of fluorescent acceptor to donor molecules determined by proximity of the two proteins. The *inserts* show examples of microscopic scans of a single osteosarcoma cell expressing (**a**) ECFP-HIF-1α and EYFP-HIF-1β fusion proteins or (**b**) ECFP and EYFP fluorophores only. The latter experiment is used to determine FRET caused by random collision and/or dimerization of the fluorophores (random FRET) which has to be taken into account when trying to prove protein–protein interaction.

4. Notes

1. To avoid hypoxic conditions due to high oxygen consumption by the cell layer, it is recommended to work with cells having confluency below 80%, i.e., within 24–36 h after seeding, before culture density becomes too high.
2. For each condition the experiments are conducted in triplicate. Use two incubators: one for normoxia (21% O_2) and the other for hypoxia (1% O_2).
3. For this and the following step, protect samples with aluminium foil to avoid photodamage.
4. This step is recommended for sensitive cell lines but not necessary for U2OS cells.

5. To reduce destruction of fluorophores during imaging experiments, one can reduce laser power or shorten scanning periods by lowering image resolution. However, reduced laser power requires higher detector sensitivity to balance the loss of signal. This can cause higher background and noise.
6. A number of plug-ins for the software "ImageJ" is available, which helps evaluate images taken for microscopic protein–protein interaction analysis (6, 7).
7. With respect to FRET analysis, ECFP-fusion proteins represent *donor*s and EYFP-fusion proteins *acceptors*. During this step imaging characteristics of the microscopic system have to be standardized.
8. In this equation it is not taken into account that during the non-radiative resonance energy transfer other relaxation processes may take place. Their amount should be negligible.
9. New developments in the field of fluorophore design promise to reduce the amount of false-positive FRET signal. New mutants of existing GFP variants eliminate hydrophobic interactions between them.

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