Chapter 19

Subcellular Dynamic Imaging of Protein–Protein Interactions in Live Cells by Bioluminescence Resonance Energy Transfer

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Abstract

Protein functions rely on their ability to engage in specific protein–protein interactions and form complexes that are dynamically regulated by stimuli. Bioluminescence resonance energy transfer (BRET) is a highly sensitive technique, which allows monitoring of interaction between two proteins: one tagged with the luminescent donor *Renilla* luciferase, the other with a fluorescent acceptor such as YFP. We adapted this method to single-cell imaging. To this aim, we tag proteins of interest, transfect cells with these fusions, and use the high-sensitivity microscopy, combined with electron multiplying cooled charge-coupled device (EMCCD) cameras and improved bioluminescence probes. We thus achieve rapid acquisition of high-resolution BRET images and study the localization and dynamics of protein–protein interactions in individual live cells.

Key words: Bioluminescence, live cell BRET imaging, subcellular localization, protein–protein interactions, bioluminescence-dedicated microscope, electron multiplying cooled charge-coupled device camera.

1. Introduction

Bioluminescence resonance energy transfer (BRET) allows real-time analysis of interactions between proteins, which are expressed in their natural location in living systems. BRET is a proximity-based assay that relies on the non-radiative transfer of energy between donor and acceptor molecules according to the Förster mechanism. The efficiency of energy transfer depends primarily on (1) the overlap between the emission

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spectrum of the donor and excitation spectrum of acceptor and (2) the distance (<10 nm) and orientation of the donor and acceptor entities (1, 2). The steep dependence of BRET on the distance between the donor and acceptor ($1/r^6$) allows monitoring of protein–protein interactions. This is achieved by attaching BRET-compatible donor and acceptor entities to the studied proteins. The energy donor is a bioluminescent enzyme such as *Renilla* luciferase (*R*luc) that generates light emission upon the addition of its substrate, whereas the acceptor is a fluorescent protein such as yellow fluorescent protein (YFP). To fully benefit from the excellent signal to background ratio provided by BRET (3), we developed BRET-based microscopy imaging, which provided the ability to study spatio-temporal dynamics of protein–protein interactions in live cells. Since it does not require sample illumination, bioluminescence imaging circumvents the problems of phototoxicity common in fluorescence microscopy, thus improving imaging in live objects (4, 5) and photosensitive tissue (6). More importantly, this methodology enables to visualize and quantify dynamics of protein–protein interactions at subcellular level in individual mammalian cells (7).

2. Materials

2.1. Cell Culture

1. Human Embryonic Kidney 293T cell line (HEK293T, Gentaur, Paris, France) (*see* **Note 1**).
2. Dulbecco's modified Eagle's medium (DMEM) (Gibco/BRL, Bethesda, MD) supplemented with 10% fetal bovine serum (FBS, HyClone, Ogden, UT).
3. Glass-bottom culture minidishes, type P35GC-0-14-C (MatTek Corporation, Ashland, MA).

2.2. Transfection

1. Plasmids: pRluc-N1 and pEYFP-N1 (PerkinElmer, Boston, MA). pDsRed-N1 (DsRed, Clontech, Saint-Germain-en-Laye, France).
2. Transfection reagent: HEPES-buffered saline (HBS, 280 mM NaCl, 50 mM HEPES [pH 7], 1.5 mM Na_2HPO_4) and 2.5 M $CaCl_2$.
3. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 1.4 mM NaH_2PO_4, 4.3 mM Na_2HPO_4, pH 7.4.

2.3. BRET

1. 2 mM Coelenterazine H (CoelH, Interchim, Montluçon, France) dissolved in pure ethanol, kept at –20°C.

2. Bioluminescence-dedicated microscope: Axiovert 200 M (Zeiss, Le Pecq, France). Plan-Apochromat 63×/1.40 Oil M27 objective (Zeiss, Le Pecq, France). Filters: Exciter HQ480/40 #44001 – emitter HQ525/50 #42017 – exciter HQ540/40 #59313 – emitter HQ600/50 #65886 – emitter D480/60 #61274 – emitter HQ535/50 nm #63944 (all from Chroma, Rockingham, VT).
3. Camera: cascade 512B (Photometrics, Evry, France).
4. Acquisition and analyses software: Metamorph software package (Molecular Devices, Downingtown, PA).

3. Methods

The catalytic oxidation of Coelenterazine H (CoelH) by the bioluminescent enzyme *R*luc results in the emission of light with maximum at 480 nm (Em480). When an appropriate energy acceptor such as the YFP is present within BRET-permissive distance from *R*luc, part of the energy can be transferred to the acceptor non-radiatively. This process leads to the excitation of the YFP and subsequent emission of light with a characteristic spectrum having maximum at 535 nm (Em535) (8) (*see* **Fig. 19.1**). One of the difficulties in establishing BRET imaging is to reliably discriminate the signal originating from the transfer of energy from that resulting from an overflow of the energy donor output into the energy acceptor detection channel. To control for the basal signal of the donor, the *R*luc fused protein (P1-*R*luc) is expressed in the absence of YFP-tagged protein. In this case, DsRed is used as a reporter of transfection to identify the cells that do not display BRET. In parallel, another pool of cells is transfected with the two proteins of interest: P1-*R*luc and P2-YFP. Twenty-four hours after transfection, these cells are pooled and cultivated for a further 24 h in the same culture dishes. After this, BRET experiments are carried out.

Once transfected cells (expressing DsRed or YFP protein and the donor protein) are identified by fluorescent imaging of DsREd or YFP, excitation light is switched off and Coelenterazine H solution is added to cells. Then emission of Rluc (at 450–510 nm, maximum at 480 nm) and YFP (510–560 nm, maximum at 535 nm) are recorded, using Em480 and Em535 filters, respectively. The ratiometric image 535 nm/480 nm reveals the subcellular localization of the interaction between P1-Rluc and P2-YFP. The cells used as BRET-negative control, i.e., expressing P1-Rluc alone, produce negligible signal.

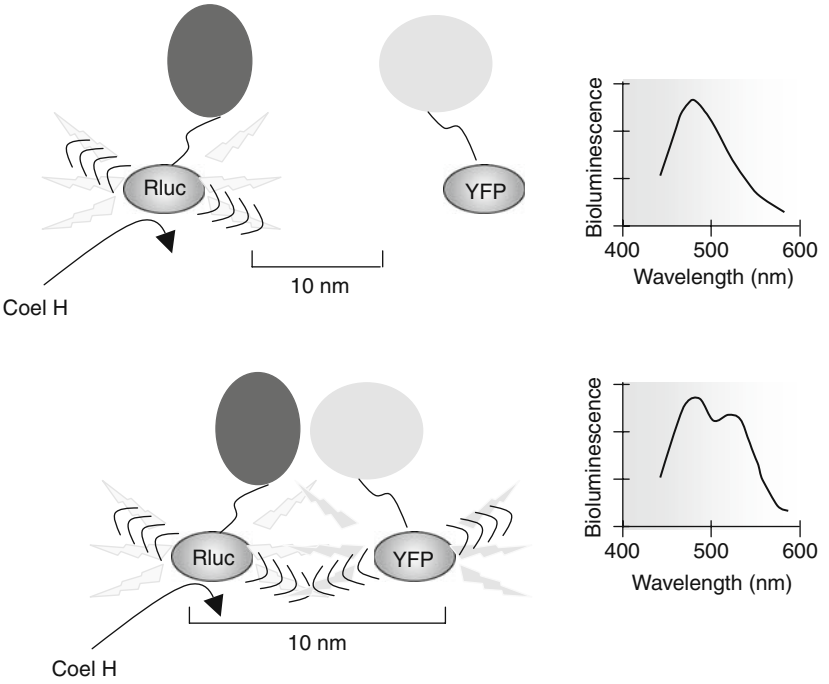

Fig. 19.1. Principle of bioluminescent resonance energy transfer (BRET). Upon binding of the two proteins, the donor and acceptor tags are brought in close proximity so that the luminescence energy resulting from the catalytic degradation of CoelH by *R*Luc is transferred to the YFP. YFP then emits fluorescence producing characteristic changes in emission spectrum. The typical effective distance between the donor and acceptor is 10–100 Å. This range correlates well with most of the biological interactions, thus making BRET an excellent tool for real-time monitoring of these interactions in living cells.

3.1. Cell Culture and Transfection

1. pRluc-N1 and pEYFP-N1 are used to fuse the DNA coding sequence of Rluc and YFP in frame with the DNA coding sequence of partner1 (P1-*R*luc) and partner 2 (P2-YFP), respectively (*see* **Note 2)** (9).
   Rluc protein:

   MTSKVYDPEQRKRMITGPQWWARCKQMNVLDSFI-
   NYYDSEKHAENAVIFLH

   GNAASSYLWRHVVPHIEPVARCIIPDLIGMGKSGKSG-
   NGSYRLLDHYKYLTA

   WFELLNLPKKIIFVGHDWGACLAFHYSYEHQDKIKA-
   IVHAESVVDVIESWDE

   WPDIEEDIALIKSEEGEKMVLENNFFVETMLPSKIM-
   RKLEPEEFAAYLEPFKEK

   GEVRRPTLSWPREIPLVKGGKPDVVQIVRNYNAYLR-
   ASDDLPKMFIESDPGFF

   SNAIVEGAKKFPNTEFVKVKGLHFSQEDAPDEMG-
   KYIKSFVERVLKNEQ –

YFP protein:

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGE-GDATYGKLTLKFICTTG

KLPVPWPTLVTTFGYGLQCFARYPDHMKQHDFFK-SAMPEGYVQERTIFFKDD

GNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILG-HKLEYNYNSHNVYIMAD

KQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGD-GPVLLPDNHYLSYQSALS

KDPNEKRDHMVLLEFVTAAGITLGMDELYK

2. For HEK293T transfection experiments, cells are seeded at a density of 2×10^6 cells per 100 mm dish and cultured for 24 h. Transient transfections are then performed using the calcium phosphate precipitation method (10). Distinct pools of cells are transfected with plasmids coding for P1-*R*luc and P2-YFP, or plasmids coding for P1-*R*luc and DsREd (transfection reporter) (*see* **Note 3**). Twenty-four hours after transfection, the two populations of transfected cells are pooled and cultured for an additional 24 h in glass-bottom culture dishes.

3.2. Imaging Set-Up

Standard inverted fluorescence microscope Axiovert 200 M is modified such that all light-emitting diodes are taken out and the light path is blocked with a 1.5-m optical fiber to limit optical interferences. The microscope is placed in a black box which protects from ambient light (*see* **Fig. 19.2**). Images are recorded at room temperature with a 63× objective. Identification of transfected cells necessitates the use of two-color detection. Exciter HQ480/40 #44001 and emitter HQ525/50 #42017 are used for YFP, whereas exciter HQ540/40 #59313 and emitter HQ600/50 #65886 are used for DsRed. BRET experiment, i.e., without photoexcitation but in the presence of luciferase substrate, requires the selection of 480 nm (filter D480/60 nm) and 535 nm (filter HQ535/50 nm) emission wavelengths. Images are collected with a cascade 512B camera equipped with an EMCCD detector, back-illumination and On-chip Multiplication Gain, which is mounted on the camera base-port of the microscope.

3.3. Identification of Cells Expressing P1-R luc Alone or P1-R luc and P2-YFP

1. Wash once the sample in glass-bottom dish containing both types of transfected cells with 200 μl of PBS and then add 200 μl of PBS.
2. Place the sample on the microscope. To identify positively transfected cells, excite the sample with appropriated filters: 540 nm excitation and 600 nm emission for DsRED;

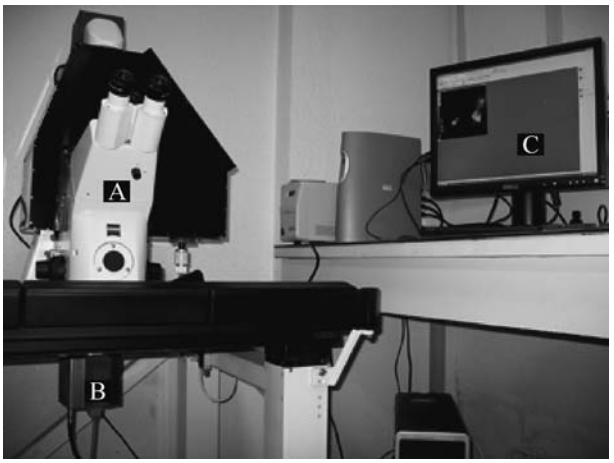

Fig. 19.2. Bioluminescence imaging set-up. (**a**) The Axiovert 200 M Microscope is an inverted fluorescence microscope, which is dedicated to bioluminescence. Thus to get rid of the ambient light pollution and collect the weak luminescent signal, the luminescent diodes were taken off, the light source deviated with an optical fiber (1.5 m long), and the microscope was installed into a *black box*. (**b**) The Cascade 512B camera is mounted on the base port of the microscope. (**c**) We use the acquisition software Metamorph.

480 nm excitation and 525 nm emission for YFP. Identify the cells expressing DsRED and P1-*R*luc alone and cells co-expressing P1-*R*luc and P2-YFP (*see* **Fig. 19.3a**). At the end of this experiment, switch off the excitation light source.

3.4. BRET Images Acquisition

1. Dilute the 2 mM CoelH stock solution in PBS to obtain 50 μl of a 100-μM CoelH solution.
2. Apply 50 μl of CoelH 100 μM in the culture dish, to get a final concentration of 20 μM CoelH, and wait for 5 min to allow diffusion of CoelH into the cell and initiation of *R*luc-catalyzed oxidation of CoelH by oxygen which produces light (*see* **Note 4**).
3. Perform sequential 30 s acquisitions (*see* **Note 5**), using the following parameters: Gain – 3950 at 5 MHz, binning – 1. Using emission filters D480/60 nm and HQ535/50 nm, obtain sets of Em480 and Em535 images, respectively. Record these raw images (*see* **Fig. 19.3b**). To follow the dynamic of the interaction between P1-Rluc and P2-YFP, subsequent acquisitions could be repeated (*see* **Note 4**).

3.5. BRET Images Analyze

1. Analyze recorded images in Methamorph software. Open the Em480 and Em535 images. In each image, define a square region (10 × 10 pixels) in the area without cells ("background region") and subtract the averaged signal

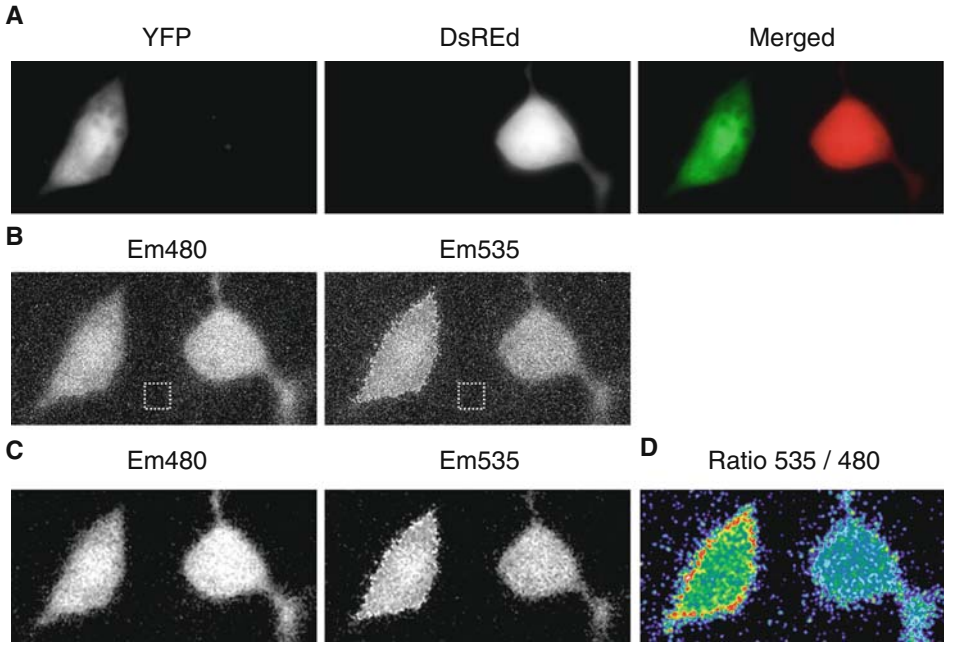

Fig. 19.3. Representative BRET images. (**a**) Identification of transfected cells. Cells are excited with a light at the appropriated wavelength for YFP or DsRED. In the same microscopic field cells express either the donor P1-*R*Luc and acceptor P2-YFP or only the donor P1-*R*Luc (+ DsRED). (**b**) Em480 and Em535 acquired 5 min after incubation with 20 μM CoelH. Em480 (*left*) corresponds to the light emitted by the P1-*R*Luc at 480 nm. Em535 (*right*) depicts light emitted at 535 nm coming from (1) the overflow of the energy donor output into the energy acceptor detection channel and (2) originating from the transfer of energy, if any. (**c**) Em480 and Em535 images obtained after subtraction of the background region (*dotted square* on **Fig. 19.3b**) and reduction of the noise with a median filter. (**d**) Ratio 535/480 image obtained by pixel-by-pixel division of the intensity emitted at 535 over 480 nm (illustrated on **Fig. 19.3c**), expressed in 255 pseudo-colors from *black* (minimum) to *red* (maximum). Note that the cell that expresses P1-*R*Luc and P2-YFP displays a much higher ratio signal than the control cell expressing only the donor. Intense BRET signals between the two partners are preferentially localized at the plasma membrane of the cell.

from this region from the raw image. Then apply a median filter to produce the Em480 and Em535 images (*see* **Fig. 19.3c**) (*see*, important, **Note 6**).

2. Generate ratiometric Em535/Em480 images by dividing the absolute intensities of each pixel of the yellow and blue images obtained at 535 and 480 nm, respectively.
3. Analyze the ratiometric images. Cells that express P1-*R*luc only donor display homogenous and relatively low ratio values resulting from donor emission leaking into the acceptor detection channel. In contrast, the cells transfected with both plasmids may display heterogenous ratio values. High values correspond to efficient energy transfer and interaction between the two proteins (*see* **Note 7**). These numerical ratios (from min to max) are processed further and visualized with a continuous 256 pseudo-color Look-Up Table, LUT (*see* **Fig. 19.3d**).

4. Notes

1. There is no restriction concerning the cell line type, provided that cells could be transiently or stably transfected. We successfully performed BRET imaging on COS cells or primary culture of hippocampal neurons.
2. The insertion of the tag should not damage the expression and function of the protein. The tag location therefore depends on the nature of the protein. The expression and function of the resulting fused protein should be compared with those of the native protein, prior to the initiation of protein–protein interaction experiments. It is worthwhile to construct several different tagged fusion proteins and choose the most efficient BRET couple. Since the BRET signal depends on the relative orientation of the donor and acceptor entities, testing several tagged couples may improve method performance.
3. DsRED has been shown to have no effect the BRET between *R*Luc and YFP (7). However, other fluorescent reporters of transfection can also be used, provided that their expression does not impair the BRET signal recorded in the presence of CoelH.
4. BRET signal usually reaches its maximum 5 min after the addition of CoelH and then remains constant for at least 25 min (7). Acquiring images within this time frame allows constant BRET values.
5. Acquisition times between 1 and 120 s can be used; however, each system has its optimum. Longer acquisition times usually improve image resolution; however, when they are too long signal-to-noise ratio can be impaired (7).
6. Median filter reduces the noise in the active image by replacing each pixel with the median of four neighboring pixel values. Therefore, median filter masks isolated pixels with ratio values in the areas having near-background signals. These areas tend to be highly variable or even undefined (division by zero).
7. To determine the minimal and maximal ratio values, identical square regions are drawn on Em535 and Em480 images on the areas of interest. The intensity of each pixel is determined and exported into Excel table. Calculate the ratio for each pixel by dividing Em535 by Em480, and find the minimal and maximal values of the ratio between which the 256-color scale will be set.

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