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A novel BRET-based genetically encoded biosensor for functional imaging of hypoxia

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ABSTRACT

Optical imaging methods, such as fluorescence, have greatly increased its impact as a monitoring technique with the development of a broad range of fluorescent proteins used to visualize many types of biological processes, such as cancer biology. Although the most popular of these proteins is the green fluorescent protein (GFP), autofluorescence due to the absorption of the exciting radiation by endogenous fluorophores and signal dispersion raises doubts about its suitability as an *in vivo* tracer. In the last years a number of groups have developed several NIR fluorescent proteins that enables real-time imaging to take place without interference from autofluorescence events allowing at the same time to take a deep view into the tissues. We therefore have outlined fluorescence–bioluminescence genetically encoded biosensor activated by the neoangiogenesis-related transcription factor HIF-1α, which is upregulated in growing tumors. At the same time, by fusing a fluorescent to a bioluminescent protein, we obtained a bioluminescence resonance energy transfer (BRET) phenomenon turning this fusion protein into a new class of hypoxia-sensing genetically encoded biosensor.

1. Introduction

Visualization of tumoral processes, either for basic research or clinical and prognosis purposes, has been recently experiencing a dramatic gain in popularity and development for obvious reasons, especially functional imaging that focuses on revealing certain activity associated to a biological process (Dunn et al., 2001; Alavi et al., 2004; Torigian et al., 2007). In the case of cancer research, the follow-up of processes such as metastasis or hypoxia, or other events intimately related to cancer biology, is clearly one of the main goals of imaging research. For this purpose, one of the most promising working alternatives to the classical diagnostic imaging techniques, such as Positron Emission Tomography (PET), Magnetic Resonance Imaging (MRI) or X-ray computed tomography (CT) to cite a just a few, is the use of optical methods that involve the emission of visible light, such as fluorescence and bioluminescence, that unlike the previous ones do not display the harming effects of the radiation on living organisms or require high-budget equipments to monitor the process (Sampath et al., 2008).

In this particular case, in the last years fluorescence came to a new position of prominence in molecular biology thanks to the cloning and use of the *Aequoerea victoria* green fluorescent protein (GFP) (Hoffmann, 2005; Prasher et al., 1992), that remains today as a widespread reporter gene. Since then, it has been developed a whole array of new fluorescent proteins with diverse excitation and emission wavelengths, making this technique very adaptable for a wide range of applications (Shaner et al., 2005).

Accordingly, the use of luciferase enzymes as reporter genes is also common. In this case, bioluminescence is produced by luciferases that catalyze 'light-emitting reactions' by oxygenating a substrate molecule, usually denominated luciferin. This process occurs in a number of living organisms, vertebrates, invertebrates and microorganisms. Unlike fluorescence where electron excitation and subsequent photon emission is mediated by light absorption, bioluminescence chemically produces singlet state species that subsequently decay emitting photon of visible light. Most used luciferases are derived from insects such as the firefly *Photynus pyralis*, marine invertebrates (*Renilla reniformis*), plants (*Gaussia princeps*) and microorganisms such as several species of vibrionaceae (Hastings, 1983). Although fluorescent light is usually brighter, with better spatial resolution and more suitable for 3D reconstruction, bioluminescent light lacks the problem of cell and tissue auto-luminescence, which results in a better signal to noise ratio for bioluminescent assays. In addition, novel fluorescent proteins can circumvent autofluorescence by shifting their excitation and emission wavelengths to the near-IR region of the spectrum, avoiding overlapping parasite emissions from tissue or

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organic compounds (Ntziachristos et al., 2003; Weissleder and Ntziachristos, 2003). These novel features are advantageous for downstream applications like bioluminescence resonance energy transfer (BRET) or genetic reporter assays.

This kind of resonance energy transfer, along with its fluorescent variant FRET, is a well-known and widespread phenomenon in proteomic procedures, specifically in determining protein–protein interactions (Pfleger and Eidne, 2006). In these assays, although the choice of the donor/acceptor pair is usually determined empirically, one of the most popular pairs is *Renilla* luciferase/GFP since they exhibit an appropriate spectral overlap of donor emission and acceptor excitation, which is one of the critical steps on the overall performance of the system. Other considerations would involve the distance between donor and acceptor besides a significant freedom of movement to allow a suitable spatial orientation for BRET to occur, and even enhancing it by inserting linkers in-between both molecules (Prinz et al., 2006).

One of the most recognizable features of a tumoral cell is the disproportionate growth that is intimately related to tumor aggressiveness and invasiveness. As a consequence of this growth, the number of blood vessels supporting the tumor rises exponentially to fulfill the exacerbated need of nutrients and oxygen. Therefore, and attending to histopathological analyses it is not uncommon to find necrotic regions inside the tumoral core, which is densely populated, in highly proliferative tumoral phenotypes. In order to alleviate this deficiency, tumoral cells elicit the formation of neovessels by stimulating neoangiogenesis. This angiogenic process is tightly regulated and results in the participation of several transcription factors, being HIF-1α one of the most important. This factor, which is stabilized in tumors with a high demand of nutrients and oxygen (Maxwell et al., 1999), exerts its transcriptional activity on several target genes that intervene in crucial processes for the tumoral phenotype such as glycolysis, apoptosis and metastasis (Bárdos and Ashcroft, 2004), making it a good marker of tumoral aggressiveness and invasion capacity in several types of tumors (Gordan and Simon, 2007; Evans et al., 2004; Furlan et al., 2007; Victor et al., 2006).

Although the development of an inducible genetically encoded biosensor is not a novelty in the field (Hoffmann, 2004; So et al., 2006; Safran et al., 2006), we describe the design, construction and characterization of a novel hypoxia genetically encoded biosensor that comprises a dual fluorescence–bioluminescence tracer capable of BRET-mediated fluorochrome excitation, overcoming the need for an external excitation source.

2. Materials and methods

2.1. Plasmid construction

The E-M-H-Luc plasmid (a gift from W.H. Suh) that combines the E-M-H enhancer plus the firefly luciferase was constructed as previously described (Lee et al., 2006). The coding sequence for mCherry (a gift from R.Y. Tsien; GenBank accession number AY678264.1) was amplified by PCR and subsequently cloned into the HindIII site of the E-M-H-Luc vector; the HindIII site was maintained by inserting compatible overhangs in both primers, thus maintaining the HindIII restriction site.

2.2. Cell culture

Human embryonic kidney HEK 293 cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Sigma) supplemented with 10% of fetal bovine serum (Fischer) and 1% L-glutamine. Cell cultures were maintained at 37 °C and 5% CO_2.

2.3. Transfections

DNA transfections were carried out according to the calcium phosphate transfection method (Graham and van der Eb, 1973). For the *in vitro* assay, cells were seeded in 24-well plates at a cellular density of 3.75×10^5 cells/well. Each group was transfected with 0.9 μg of E-M-H-mCherry-Luc and different pcDNA3/pcDNA3-HIF-1α ratios up to 0.15 μg, ranging from 0.01 μg (lowest concentration) to 0.32 μg of pcDNA3-HIF-1α (highest concentration). For the luciferase assay, cells were seeded in 12-well plates at a cellular density of 7.5×10^5 cells/well. Each group was transfected with 1.8 μg of E-M-H-mCherry-Luc and different pcDNA3/pcDNA3-HIF-1α ratios up to 0.3 μg, ranging from 0.02 μg to 0.64 μg of pcDNA3-HIF-1α. In addition, 2 μg of CMV-EGFP-Luc were transfected in the control group and 0.4 μg of pCMVβ (carrying the β-galactosidase gene) in all groups as a transfection control. As for the *in vivo* assay, cells were seeded in 10 cm plates for transfection assays at a cellular density of 3×10^6 cells/plate. Cells were transfected with 7.5 μg of E-M-H-mCherry-Luc along with 0.5 μg of pcDNA3-HIF-1α for the cell population with the 'activated system' described above.

2.4. Luciferase assay

Cell cultures were lysed 48 h upon transfection with 100 μL of 1× Passive Lysis Buffer (Promega) for 20 min at room temperature. 50 μL of each cell lysate were mixed with 35 μL of Luciferase Activity Buffer (25 mM glycylglycine pH 7.8, 15 mM phosphate buffer pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 2 mM ATP, 1 mM DTT) and 25 μL of 1 mM solution of the substrate D-luciferin (L9504, Sigma–Aldrich). Luciferase activity was measured with a luminometer Lumat LB 9507 (Berthold Technologies). To assess transfection efficiency, luciferase activity data were normalized by performing a β-galactosidase activity assay. 50 μL of each cell lysate were mixed with 250 μL of Z buffer (0.06 M $Na_2HPO_4{\cdot}7H_2O$, 0.04 M $NaH_2PO_4{\cdot}H_2$, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M 2-mercaptoethanol) and 60 μL of a 4 mg mL^{-1} solution of *o*-nitrophenyl-β-D-galactopyranoside (ONPG). Samples were incubated at 37 °C for 1 h and then β-galactosidase activities were measured with a spectrophotometer BioMate 3 (Thermo Spectronic, Winsconsin) at 420 nm. Each assay was replicated at least three times for every experimental group.

2.5. Spectrophotometric profile of the fusion protein

λ_{exc} and λ_{em} of mCherry-luciferase were determined in a FluoroMax-3 (ISA Jobin Yvon-Spex, Edison, New Jersey). Measures were obtained from cell lysates from HEK 293 cells transfected as described above.

2.6. Confocal microscopy

Cells were fixated 48 h upon transfection by incubation in 3.5% paraformaldehyde for 10 min at r.t. and subsequently 5 min at 4 °C. Samples were mounted in UltraCruz Mounting Medium (Santa Cruz, sc-29491). Images were obtained in a Spectral Confocal Microscope Leica TCS-SP2 (Leica, Mannheim, Germany) with a spectral range of detection from 400 nm to 850 nm.

2.7. Fluorescence–bioluminescence in vivo assays and animal care

Upon sedation with isoflurane using the XGI-8 Gas Anesthesia Unit (Caliper Life Sciences, USA) fluorescence and bioluminescence data were registered with an IVIS Spectrum (Caliper Life Sciences, USA) in 30 s expositions during 30 min. For bioluminescence and BRET assays SCID mice (Charles River Laboratories, USA)

Fig. 1. The scheme shows the design of the genetically encoded biosensor: a regulatory module formed by the E-M-H enhancer and the SV40 minimal promoter, and the tracer module comprised by the fusion protein formed by the mCherry fluorophore and the firefly luciferase.

were injected intraperitoneally with a single dose of D-luciferin (L9504, Sigma–Aldrich) dissolved in sterile PBS. Mice were housed in specific pathogen-free (SPF) conditions, following Federation of European Laboratory Animal Science Associations (FELASA) guidelines for animal housing and according to the USC Bioethic Board regulation, passed on September 23rd, 2003.

3. Results and discussion

3.1. Design and construction of the hypoxia genetically encoded biosensor

Our genetically encoded biosensor comprises a regulatory module, that contains a transcriptional enhancer able to bind the alpha subunit of the HIF-1 transcription factor, and a dual tracer formed by a fusion protein of a NIR fluorophore and the firefly luciferase. Several fluorescent proteins were considered on the basis of their excitation and emission wavelengths. Although initially mCherry and mPlum were tested as prospective fluorochromes, mPlum was eventually discarded due to its low brightness (data not shown; Shaner et al., 2004). On the other hand, mCherry has an excitation wavelength of 585 nm that makes this fluorophore suitable for being excited by bioluminescent light (575 nm).

As for the regulatory moiety of the construct, we employed as a HIF-1α sensor a chimeric combination of the (Egr-1)-binding site (EBS) from the Egr-1 gene, the metal-response element (MRE) from the metallothionein gene, and the hypoxia-response element (HRE) from the phosphoglycerate kinase 1 gene. This chimeric enhancer has been described to trigger a transcriptional response to a greater extent and increases hypoxia responsiveness when compared to the hypoxia-response element alone (Lee et al., 2006). A SV40 minimal promoter is located downstream of the chimeric enhancer E-M-H.

The construction containing the fusion protein formed by mCherry and the firefly luciferase appears outlined in Fig. 1.

3.2. Spectrophotometric characterization of the fluorescent–bioluminescent fusion protein

We next checked the performance of the fusion protein by assessing the activity of both the fluorochrome and the luciferase enzyme. The integrity of the aminoacidic environment of the fluorescent proteins is crucial in order to maintain its activity (Baird et al., 2000; Ai et al., 2007; Shu et al., 2006). For that purpose, we tested the *in vitro* fluorescent activity of the fusion protein by obtaining their spectrophotometric profiles. Fig. 2a shows that mCherry retains the same wavelengths of excitation and emission that the ones reported before (Shaner et al., 2005), indicating that fusing the luciferase and mCherry together did not affect the performance of the fluorescent protein *in vitro*.

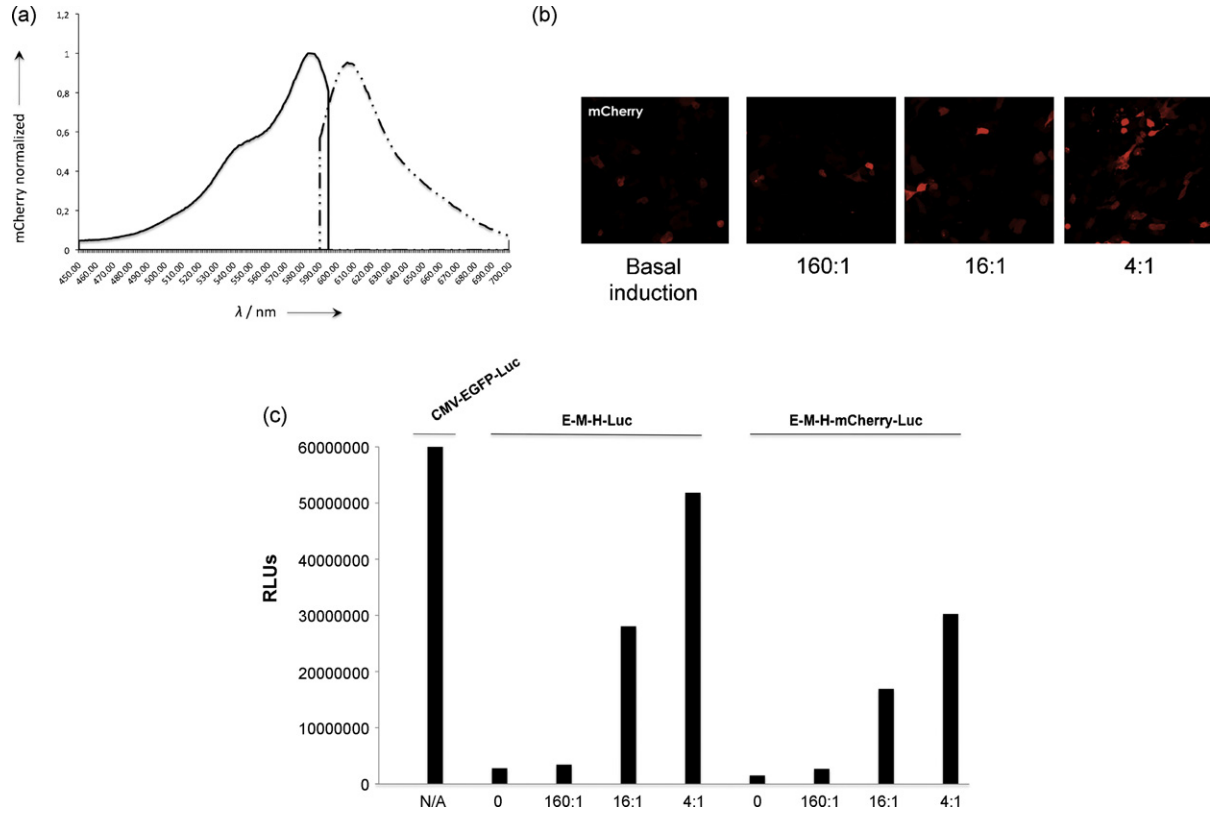

Fig. 2. (a) Spectrophotometric profile of mCherry in the fusion protein. Solid line represents mCherry excitation while dashed line represents mCherry emission; (b) fluorescence images of the mCherry protein. Concentrations of HIF-1α are given in E-M-H-mCherry-Luciferase:pcDNA3-HIF-1α ratios; (c) luciferase activity of the fusion protein for E-M-H-Luciferase and E-M-H-mCherry-Luciferase. Concentrations of HIF-1α are given in E-M-H-mCherry-Luciferase:pcDNA3-HIF-1α ratios.

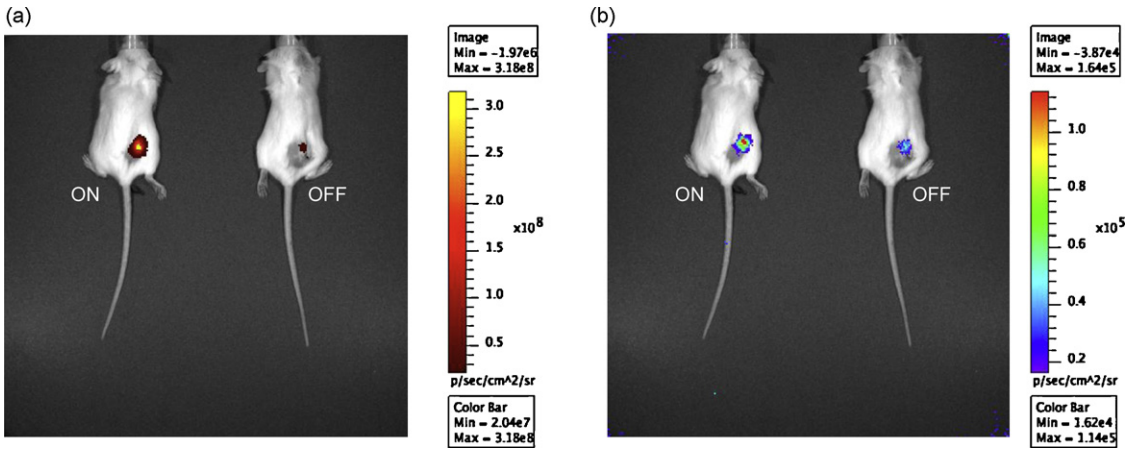

Fig. 3. *In vivo* fluorescence and bioluminescence measure in a xenograft implanted subcutaneously in SCID mice; (a) mCherry fluorescence in the 'activated system' (ON) in HEK 293 cells transfected with E-M-H-mCherry-Luciferase and pcDNA3-HIF-1α and 'non-activated' system (OFF) in cells transfected with E-M-H-mCherry-Luciferase and pcDNA3; (b) luciferase activity in the 'activated system' (ON) and 'non-activated' (OFF) system.

3.3. Dose–response analysis of the genetically encoded biosensor to HIF-1α

Subsequently, we wanted to corroborate these data by testing the functionality of the cloned fluorescent protein. As Fig. 2b shows, we transfected our construction along with increasing amounts of the HIF-1α transcription factor into the cell line HEK 293. As expected, our data indicate that the system is proportionally responsive to the amount of transcription factor transfected in each case. Curiously, although the basal induction (i.e. induction by endogenous levels of HIF-1α) seems to be higher than the lowest amount of HIF-1α, the luciferase assay proves that the response elicited in both cases is similar, thus indicating that the threshold of detection corresponds to the amount of HIF-1α transfected in this case.

Similarly, the luciferase activity of the genetically encoded biosensor, shown in Fig. 2c, appears to be increasingly higher as the concentration of the transcription factor rises. The empty vector without mCherry, included as a control, shows a similar proportional response but with a significantly higher maximum value than the genetically encoded biosensor. As means of transfection normalization, the data obtained were normalized against the β-galactosidase activity of each group, as described in Section 2.

Taken together, these data show that this constructs effectively binds to the transcription factor HIF-1α and elicits the transcription of the fusion protein mCherry-luciferase. Also, the fluorescent properties and the luciferase activity of this fusion protein remain unaltered although its intensity in this case seems to be lower than the vector containing both the response element and the luciferase. This difference will be investigated further to assess whether it is caused by the transference of resonant energy between the luciferase and the fluorescent protein or the fusion protein resulted in a hindered luciferase with a reduced ability to catalyze the bioluminescent reaction.

3.4. In vivo analysis of the genetically encoded biosensor performance

We next investigated whether or not the *in vitro* performance of the system could be replicated and measured in an *in vivo* environment. In an analogous manner, we co-transfected the cell line HEK 293 with our construction and either pcDNA3-HIF-1α or pcDNA3 as control. Thus, we obtained two populations of cells either with the activated system or 'basal state' cells (transfected only with the genetically encoded biosensor) injected subcutaneously in the hindquarters of SCID mice as Fig. 3 indicates. Fig. 3a shows the *in vivo* fluorescent light measured 24 h upon injection of both populations. Attending to these data, it is clear that the system remains active and its intensity is significantly higher in the cell population harboring the genetically encoded biosensor with the transcription factor HIF-1α than the cells displaying basal activation.

In the same way, luciferase activity was also measured *in vivo* (Fig. 3b). Upon administration of luciferin, we observed a similar outcome; both cell populations display bioluminescent light emission but with a higher intensity in the cells with the activated system. Taken together, these data demonstrate that this hypoxia-sensing genetically encoded biosensor is able to proportionally induce the synthesis of the mCherry-luciferase fusion protein depending on the concentration of HIF-1α. Additionally, this response is enabled both *in vitro*, in HEK 293 cells transfected with increasing concentrations of HIF-1α, and *in vivo* xenografts of these transfected cells, as shown in Figs. 2 and 3.

3.5. In vivo bioluminescence resonance energy transfer in near-infrared fluorescent probe for detecting hypoxia

As discussed above, there seems to be a fall in the luciferase activity of the system when compared to the parental vector that contains only the response element and the luciferase. We wanted to confirm that this difference is caused by the transference of bioluminescent resonant energy or BRET-mediated mCherry excitation in the absence of an external source. Consequently, we first tested whether this transference was occurring *in vitro* by comparing the spectrophotometric profiles of the luciferase of the parental vector (without mCherry) with the fusion protein (mCherry-luciferase). As Fig. 4a shows, the luciferase alone displays a maximum value at the expected wavelength of 575 nm. However, although the fusion protein displays a peak at this wavelength, it also displays a lower second peak at a wavelength corresponding with the emission maximum of mCherry, As we mentioned above, the fall of bioluminescent signal is also observed in this case and that would be consistent with the existence of BRET as suggested by the second emission peak.

Subsequently, we next investigated whether this BRET transference could be also detected *in vivo*. For that purpose, we tried to quantify the emission of the activated system in the presence of luciferin and at the same time avoiding the interference of any external excitation source by blocking the excitation filter. Since this process critically depends on the light emitted by the reaction catalyzed by the luciferase, we registered the fluorescence emission

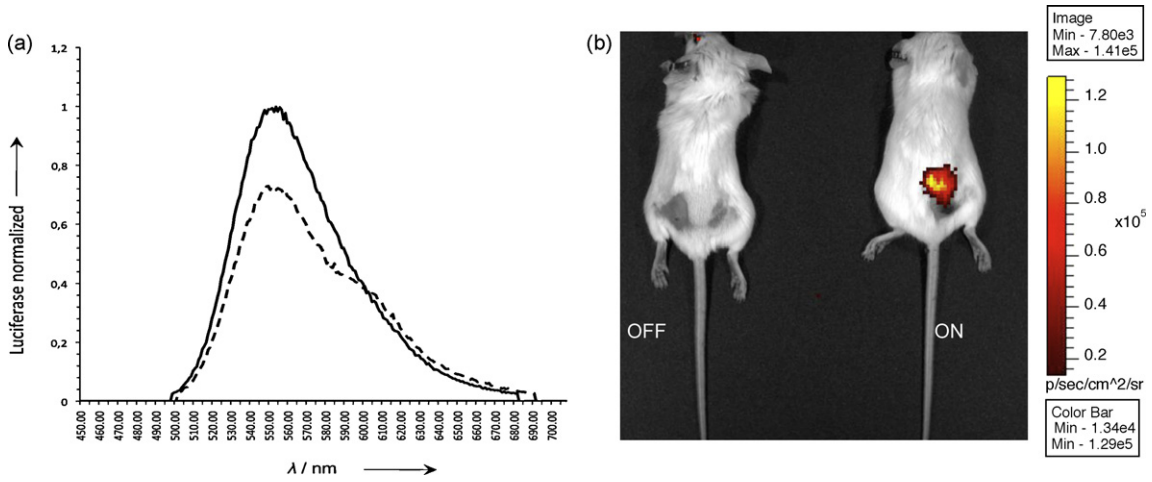

Fig. 4. (a) *In vitro* BRET performance of the genetically encoded biosensor. Solid line represents luciferase activity of the E-M-H-Luciferase vector while dashed line represents luciferase activity of the E-M-H-mCherry-Luciferase vector; (b) *in vivo* BRET performance of the system.

over 30 min upon the injection of the luciferase substrate. Fig. 4b shows the peak emission of BRET in SCID mice, reached at 15 min upon luciferin injection.

4. Conclusions

In conclusion we have described the designing and development of a hypoxia-sensing system with both NIR fluorescent and bioluminescent properties. This genetically encoded biosensor is induced by the hypoxia transcription factor HIF-1α. We have demonstrated that when the genetically encoded biosensor is present, this factor binds to the response element located in the regulatory module of the construction inducing the transcription of the fusion protein. This dual tracer displays a proportional response to the quantity of HIF-1α present. We have also described the excitation and emission of the mCherry fluorochrome, both *in vitro* and *in vivo*, through a BRET phenomenon elicited by the firefly luciferase emission. The combination of near-infrared fluorescence and bioluminescence paves the way for future developments that take advantage of BRET, especially in the follow-up of processes where an internal excitation source helps to overcome technical problems such as tissue autofluorescence and weak tissue penetration of wavelength excitation light.

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