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Title page

TECHNICAL NOTE

Screening for protein-protein interaction inhibitors using a Bioluminescence Resonance Energy Transfer (BRET) based assay in yeast

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Abbreviations:

ATP: Adenosine Tri-Phosphate; BRET: Bioluminescence Resonance Energy Transfer; CDK: Cyclin Dependent Kinase; CML: Chronic Myelogenous Leukaemia; FKBP12: 12kDa FK-506 binding protein; FRET: Förster/Fluorescence Resonance Energy Transfer; HDM2: Human Double Minute 2; HTS: High Throughput Screening; mBRET: milliBRET; NCoR: Nuclear receptor CoRepressor; NLuc: Nano Luciferase; OD: Optical Density; P2I2: Protein Protein Interaction Inhibitor; PBS: Phosphate Buffered Saline; PPI: Protein-protein interaction; RLuc: *Renilla* Luciferase; SD: Synthetic Dextrose medium; SR: Synthetic Raffinose medium; TGFβR: Transforming Growth Factor β Receptor; TR: T3/Thyroid hormone Receptor; YFP: Yellow Fluorescent Protein.

Abstract

The Bioluminescence Resonance Energy Transfer (BRET) technology is a widely used live cell-based method for monitoring protein-protein interactions as well as conformational changes within proteins or molecular complexes. Considering the emergence of protein-protein interactions as a new promising class of therapeutic targets, we have adapted the BRET method in budding yeast. In this technical note, we describe the advantages of using this simple eukaryotic model rather than mammalian cells, in order to perform high-throughput screening of chemical compounds collections: genetic tractability, tolerance to solvent, rapidity, no need of expensive robotic systems. Here the HDM2/p53 interaction, related to cancer, is used to highlight the interest of this technology in yeast. Sharing the protocol of this BRET-based assay with the scientific community will extend its application to other protein-protein interactions, even though toxic for mammalian cells, in order to discover promising therapeutic candidates.

Introduction

Protein-protein interactions (PPIs) govern virtually all cellular processes and thus offer a tremendous panel of opportunities for therapeutic intervention. Targeting PPIs instead of single proteins provides a means to increase drug specificity and efficacy. Since the interface of a particular PPI is formed by the combination of interacting domains of two particular proteins, it will display a higher level of uniqueness in comparison to, for instance, the catalytic pocket of an enzyme, which is often well conserved throughout a whole enzyme class. As examples, all of the active human protein kinases use ATP to phosphorylate their substrates. On the market since 2001, imatinib mesylate (Gleevec, Novartis, Basel, Switzerland), which targets the ATP-binding site of the tyrosine kinase Bcr-Abl, was the first targeted therapy developed for chronic myelogenous leukaemia (CML). During cancer treatment, point mutations may arise on a single cavity, to escape drugs targeting the catalytic pocket while keeping the enzymatic activity. Concerning CML treatment with imatinib mesylate, the main cause of therapy failure is related to mutations affecting principally the ATP-binding cleft and notably a key residue, termed the “gatekeeper”, located at the back of this pocket[1]. To circumvent such resistance phenomenon, one potential therapeutic strategy can be the inhibition of protein-protein interactions (PPIs) required for activation of signaling pathways downstream of Bcr-Abl (strategy explored in [2]). Indeed, eluding a protein-protein interaction inhibitor (P2I2) while preserving the interaction would require a second compensatory mutation in the binding partner, which would be much less probable. The challenge is thus to discover small molecules that disrupt protein-protein complexes. To this end, robust, facile high-throughput methods, preferentially in a living cellular context, are needed (screening assays are reviewed in [3]).

The yeast-based *Bioluminescence Resonance Energy Transfer* (BRET) assay described herein offers a powerful method to discover small-molecule inhibitors of protein-protein interactions. In this technical note, we use the HDM2/p53 interaction (of particular interest for cancer therapy[4]) and its small-molecule inhibitor Nutlin-3 to exemplify this scalable method.

BRET technology in molecular screening

BRET is a naturally occurring phenomenon that can be observed in the sea pansy *Renilla reniformis* and is similar to an existing method for assessing PPI, the Förster or more commonly called fluorescence resonance energy transfer (FRET). In FRET, one fluorophore (the “donor”) transfers its excited-state energy to another fluorophore (the “acceptor”), which emits fluorescence at a longer wavelength. In both methods, the donor and acceptor are genetically or chemically fused to candidate proteins or compounds. In BRET, a luciferase is used as the energy donor to avoid the consequences of donor excitation in FRET (for instance the damage of tissues by the excitatory light, photobleaching and simultaneous excitation of the acceptor by the donor excitatory light). In the presence of its substrate, bioluminescence from the luciferase occurs and the transfer of energy leads to the excitation of the acceptor fluorophore, if the donor and acceptor are close enough (<10nm), which can occur upon a molecular interaction between the fused proteins. BRET is a highly versatile technique that can be used to measure protein interactions *in vitro* (using purified proteins, crude cell membranes or other cell fractions), in cultured cells and *in vivo*[5]. Based on the BRET method, different screening assays have been designed (reviewed in [6]).

Why is budding yeast a suitable alternative model to setup a cellular screening assay and to maximize hit rates?

The unicellular baker's yeast *Saccharomyces cerevisiae* is a proven model for fundamental and applied research. For example, basic cellular processes occurring in human cells are well conserved in yeast (*e.g.* the control of the cell division cycle). This safe organism is also genetically well defined, since its entire genome was sequenced in 1996. Moreover, yeast growth and division can be precisely controlled, and different strategies can be adopted to maximize hit rates in yeast-based assays: (i) Enhancing the limited cell permeability to small molecules by alteration of yeast membrane composition. Classically, *erg6* gene mutation (involved in the ergosterol biosynthesis, one of the components of fungal membranes) was found to increase permeability to a growing list of chemical compounds, in particular to small lipophilic drugs[7]. (ii) Screening molecules against non-preformed complexes[3,8]. It is now considered essential that small molecules have to be delivered to the cellular system before the synthesis of one or the two interacting proteins. The use of inducible promoters, such as the *GAL1* promoter, enables the small molecules to first interact with one of the two partners, prior to protein complex formation. Indeed, in a BRET study conducted in mammalian cell lines, where cells were transiently transfected with fusion genes to monitor the HDM2/p53 interaction, the inhibitor Nutlin-3 was not able to completely disrupt a preformed complex[9]. In our experimental set-up the use of inducible protein expression allowed to completely inhibit HDM2/p53 interaction (see section "Monitoring the Effect of a P2I2 Using a BRET Based Assay in Yeast: The Inhibition of the HDM2/p53 Interaction by Nutlin-3 as Proof of Concept"). (iii) The development and selection of stable strains in which the interacting proteins can be expressed, that can be achieved in 1 to 2 weeks. This is in sharp contrast with

mammalian cells, where obtaining stable lines is classically time consuming and sometimes arduous. (iv) The possibility to express mammalian proteins involved in toxic or death response in mammalian cells. (v) The rapid expansion of yeast compared to mammalian cells allowing to obtain of the proper amount of cells needed to perform HTS experiments in less time. Besides all technical benefits described above this is one of the main advantages of this model. Indeed, proteins implicated in the cell cycle or apoptosis control may impair the development of such screening assay in mammalian cells. Nevertheless, some limitations exist when using yeast as (i) the reduced sensitivity to some classes of compounds due to efficient drug efflux pumps, (ii) when overexpressed in yeast, some human proteins can be toxic (*e.g*. some tyrosine kinases), (iii) some protein interactions depend on post-translational modifications, that do not exist in yeast and cannot be screened (*e.g*. absence of tyrosine phosphorylations in yeast) and (iv) some protein interactions might involve folding/conformations that depend on chaperones which may not exist, or may not function in yeast. However, easier genetic manipulations of yeast allowed the development of strains expressing human genes counteracting cell death. These models may thus represent a major advantage of yeast for such screening purpose. The study of CDK5/p25 interaction highlights this key advantage of yeast[8]. Indeed, p25 is a 25-kDa pathological proteolytic fragment of p35, one of the physiological regulatory subunit of CDK5 kinase. Previous study has shown that even low basal level expression of p25 is toxic for mammalian cells, and thus stable cell lines were obtained only if the tau protein is constitutively co-expressed[10]. In yeast, we were able to establish a stable inducible p25 yeast strain that doesn't require tau co-expression as suitable model to study the CDK5/p25 interaction[8].

Here, we detail a robust and scalable BRET based cellular screening assay developed in the budding yeast, which supports the discovery of inhibitors of protein-protein interactions.

Materials and methods

Chemicals. D(+)-Galactose, D(+)-Raffinose were purchased from ACROS Organics (Geel, Belgium). Yeast nitrogen base without amino acids and Bacto peptone were purchased from BD Biosciences (Franklin lakes, NJ). D(+)-Glucose, Bacto yeast extract, Dimethylsulfoxyde (vehicle control), 3,3'5-Triiodo-L-thyronine sodium salt (T3), Nutlin-3, Nutlin-3a, FK-506 monohydrate and the different amino acids complements were purchased from Sigma (St Louis, MO).

Reagents. Coelenterazine h was purchased from Interchim (Montluçon, France) and Nano-Glo® Luciferase Assay Substrate from Promega (Madison, WI). *erg6* yeast mutant strain was obtained from Euroscarf no.Y00568 BY4741; MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0; YML008c::kanMX4 (www.euroscarf.de). The antibodies used are anti-GFP (#ab290; Abcam) and anti-RLuc (#MAB4400; Millipore).

Equipment. Mithras LB940 Fluorometer/ Luminometer (Berthold Technologies, Bad Wildbad, Germany) microplate reader was used with the following emission filters:

- RLuc/NLuc; Counting Time: 2.00 seconds; Emission Filter (#39450): 480nm (±10nm);
- eYFP; Counting Time: 2.00 seconds; Emission Filter (#39451): 530nm (±12.5nm).

Measurement Operation is performed by well and each plate is read three times in a cycling manner.

BRET calculation. BRET ratio was calculated by dividing the signal measured at 530nm by the signal measured at 480nm. Then, BRET signal, was calculated as the BRET ratio subtracted by the BRET background ratio (obtained when the donor protein was expressed alone) and multiplied by 1000 to express results in milliBRET (mBRET):

BRET = (BRET ratio – Background BRET ratio) X 1000, detailed in [6].

Statistical analysis. Data were expressed as mean ± SD, or as percentage ± %RSD (percent relative standard deviation: SD X 100 / mean). Statistical analyses were done by Student's *t* test and significance levels used are: *p<0.05; **p < 0.01; ***p<0.001; n.s., not significant.

Vector constructions. The centromeric vectors p415 (*LEU2, GAL1* inducible promoter) and p416 (*URA3, GPD* constitutive promoter) were used to express the chimeric proteins. *TRP1* selectable marker gene cannot be employed with *erg6* mutant strain, because *trp1-erg6* double-mutant is synthetic-lethal. Donor (HDM2-RLuc or HDM2-NLuc) proteins were cloned into p415 and acceptor proteins (p53-YFP and p53m(F19A)-YFP) were cloned into p416, see Table1 and Suppl. Fig. for more details. Both C- or N-terminal fusion vectors are available upon request.

Results and Discussion

PPIs control many cellular processes, including metabolic cycles, DNA transcription and replication, enzyme activity, different signaling cascades and other processes. The importance of PPIs justifies the development of new powerful methods to

understand the role of such interactions and to discover inhibitors. The protocol described below is based on the BRET technology, a non-radiative energy transfer, which can be applied to monitor proteins interactions and to identify potential inhibitors. The innovation of this HTS assay lies in the use of the budding yeast *S. cerevisiae* rather than mammalian cells, which facilitates, expedites and reduces the cost of assay development.

Feasibility study and assay optimization.

A schematic representation of the BRET-based assay is provided in Fig. 1A. As depicted, energy transfer occurs when the donor and the acceptor, respectively fused to the interacting proteins, are 1-10nm apart. To monitor the BRET signal, two yeast strains are needed: one strain expressing the energy donor alone (for background detection, here HDM2-Luc) and the other expressing both the donor and the acceptor fusion proteins (here HDM2-Luc and p53-YFP). An advantage in working with yeast is the possibility to modify their membrane permeability to favor the penetration of small drug-like molecules inside the cell. An *erg6* strain is thus chosen for the development of the BRET-based screening assay in order to increase the hit rate. Moreover, to screen against a non-preformed complex, the protein expression of the energy donor is placed under the control of an inducible *GAL1* (galactose regulated) promoter. Once the yeast transformed, expression of both chimeric proteins should be verified by SDS-polyacrylamide gel electrophoresis followed by immunoblotting (Fig. 1B). Induction using galactose is an effective way to control gene expression as shown for HDM2-RLuc.

To obtain a significant BRET signal, donor and acceptor groups should be in closed proximity but an absence of the BRET signal does not necessarily mean that there is

no interaction, it could be due to a non-optimal orientation of the BRET partners. To circumvent this problem, it is crucial to test different combinations in which the proteins of interest are fused to donor and acceptor at the C- or N-terminal extremities and using several linker peptides with flexibility (for example, GS or GGS repetitions). Indeed, one of the orientations would be favorable over the others and lead to maximal BRET signal. In our case, donor and acceptor groups fused at the C-terminal extremities is considered for screening.

The optimal expression level of donor fusion protein should be evaluated by measuring the BRET signal in response to increasing galactose concentration (from 0.001% to 2%) and time of induction (up to 6 hours). This evolution of the BRET signal is protein interaction-dependent and must be monitored for each new PPI. The conditions to choose for the screening are those that produce the highest measured BRET signal. The use of other donor proteins can be envisaged. NLuc (NanoLuc®, Promega) is a smaller and brighter luciferase (19.1kDa) than RLuc. It presents a high thermal stability, a strong activity over a broad pH range, and its emission peak (465nm) is suitable for BRET assay. Moreover NLuc signal is stable for long time (up to 1 hour). Therefore NLuc was tested in the same way than RLuc. In the case of HDM2/p53, the highest BRET signal was measured after 2 hours, in presence of 2% galactose (Fig. 1C,D). As expected, the NLuc signal intensity is higher compared to RLuc (almost 20 folds in our hands), at all inducer concentrations assayed (*not shown*). BRET signal was 20-50mBRET higher with NLuc vs RLuc at low inducer concentration (≤0.05%), while it was comparable when the donor saturated the acceptor (galactose concentration >0.1%) (Fig. 1D). NLuc can thus be employed usefully to overcome problems of low expression levels of some heterologous proteins.

Another requirement to validate a screening assay is to demonstrate the specificity of the BRET signal, which can be done either by using a mutation that destroy the interaction, or by performing a donor saturation assay. For this purpose the F19A mutation, involving a key residue for the interaction with HDM2[11], was introduced in p53. It caused a complete loss of the BRET signal, thus confirming the specificity of the signal obtained using wild-type proteins (Fig. 1E). Otherwise, when information about a point mutation abolishing PPI is lacking, non-specific BRET signal, due to random collisions between donor and acceptor, can be measured by co-transforming yeast with YFP alone (cloned in the same vector of the acceptor fusion protein) and donor fusion protein.

In the classical donor saturation assay, the BRET signal is measured in response to increasing amounts of acceptor (achieved by increasing inducer concentration), while the donor is kept constant. The expected result for a specific BRET signal is a hyperbolic curve. Nevertheless, inducible donor fusion protein is preferred for HTS as besides the possibility to directly verify that the expression of the protein is well induced, another advantage of inducing the donor instead of the acceptor is the ability to reach the maximal BRET signal upon induction. Indeed, donor saturation by the acceptor occurs quite instantly as huge amount of the acceptor are already present due to its constitutive expression.

Monitoring the effect of a P2I2 using BRET-based assay in yeast: the inhibition of HDM2/p53 interaction by Nutlin-3 as proof of concept.

p53, also called the guardian of the genome, is negatively modulated by HDM2. Disrupting the HDM2/p53 interaction may thus offer a new strategy for cancer

therapy. To this end, in 2004, the chemical compound Nutlin-3 was discovered using Surface Plasmon Resonance[12].

We have examined the effect of Nutlin-3 on the HDM2-p53 BRET signal in our assay. The BRET signal decreased in a dose-dependent manner (Fig. 2A). As shown in Fig. 2B, the inhibitory effect of Nutlin-3 and its enantiomer Nutlin-3a[12] has been also verified using NLuc as donor. As expected, the Nutlin-3a, known to be more potent on the HDM2/p53 inhibition than Nutlin-3, showed a higher inhibitory effect (Nutlin-3 IC_{50} = 28.6 µM, Nutlin-3a IC_{50} = 8.8 µM) on HDM2/p53 interaction.

The effect of other known PPI inhibitors was also evaluated on HDM2/p53 interaction using this BRET-based assay. As shown in Fig. 2C, no effect was observed on the BRET signal using FK506, T3, or tamoxifen, which inhibit the FKBP12/TGFβR, NCoR/TRα and CDK5/p25[13] interactions, respectively.

Prior to starting a large screening campaign, pilot screenings are used to predict if it would be feasible in a high-throughput setting. For this purpose, the calculation of a coefficient called *Z*'-factor is recommended (see [14] for details). This statistical dimensionless parameter is essential to evaluate and validate the quality of HTS assays. The *Z*'-factor is defined in terms of four parameters: the means and standard deviations of both the positive (p) and negative (n) controls (μ_p, σ_p and μ_n, σ_n). Usually, the negative control is determined without the tested enzyme or using non-induced conditions. Considering this yeast-based screening assay, it is experimentally inconceivable to use the same yeast strain to obtain a "non-induced" negative control. Indeed, in non-induced condition (yeast growing in raffinose-based media without galactose), no reliable BRET signals can be achieved. We therefore decided to adapt the calculation using a known inhibitor, Nutlin-3. To characterize

this estimated coefficient (named Z-yBRET factor on Fig. 2D), we prepared a 96-well plate and measured the BRET signal obtained with the compound vehicle (DMSO) (p, positive controls) or 20µM of Nutlin-3 (n, negative controls), on the HDM2/p53 interaction. The formula used for the calculation is reported on Fig. 2D, and is similar to the one of Z'-factor[14]. According to the scatter plot obtained, we observed a significant difference between the two sets of samples (separation band), confirming that this method is adapted for screening, with a calculated factor coefficient of 0.67 (Fig. 2D). This value indicates a suitable difference between maximal signal and inhibited values (or background) together with low variability. Moreover, when the more efficient inhibitor Nutlin-3a was used, the calculated factor coefficients were 0.70 and 0.85, using RLuc or NLuc as donor respectively (*not shown*).

Nevertheless, if any P2I2s are already described for the targeted protein-protein interaction, values obtained for strains transformed with Luc-fusion protein and YFP alone can be also used as negative control. For our model interaction, HDM2/p53, we obtained a factor coefficient of 0.80 (using RLuc as donor protein) and 0.94 (when using NLuc) (*not shown*). These results indicate that this yeast-based screening method is suitable for use in a full-scale, high-throughput screening.

Screening

Day 1, 10-20 yeast colonies were picked from selection plates (SD-Ura-Leu media, SD: Synthetic Dextrose medium) and inoculated in liquid media (See Suppl. Data for a step-by-step detailed protocol). The two yeast strains, transformed by the donor and acceptor expression vectors or the donor alone and the empty acceptor plasmid p416GPD were grown overnight (12h) at 29°C in 5 mL of liquid SR-Ura-Leu (SR for Synthetic Raffinose medium). Raffinose 2% (final concentration) is a carbon source

that neither represses nor induces the Gal promoter and that allows prompt donor expression upon galactose (inducer) addition. Day 2, the cultures were diluted to an optimal OD of 1 (A_{600nm} = 1) suitable for screening in fresh SR-Ura-Leu. Then, the 96-well plates were filled with 36µL of yeast culture (according to the plate map, see Fig. 3). As depicted, the first and 12^{th} columns of the 96-well plate were dedicated to controls and eight wells were filled by the control strain expressing the BRET donor only (used for the background BRET ratio calculation). Then, 0.4µL of the tested molecules, at the appropriate concentration, was added. Alternatively, and depending on liquid handling systems available in the lab, yeasts can be added in 96-well plate already containing the tested compounds. We experimentally observed that yeast tolerates up to 3% of DMSO (solvent for chemical compounds). 80 different compounds can be analyzed per 96-well plate. Finally, to induce the *GAL1* promoter, 3.6µL of galactose were added from a 10X stock solution (20% or otherwise optimized) and the plate was placed in a shaking platform for 2 hours at 29°C. We used here the induction time optimized as described in section "Feasibility Study and Assay Optimization" and reported on Figure 1C. A few minutes before the end of the incubation, a fresh dilution of luciferase substrate was prepared. For RLuc donor substrate, coelenterazine h was solubilized in ethanol and diluted in PBS (Phosphate Buffered Saline) to 5µM final concentration in the wells. The Nano-Glo® Luciferase Assay Substrate was first diluted in PBS and used as 5000X final dilution in the wells. The multi-well plate was then loaded in the BRET reader and BRET values were determined as described in section "BRET Calculation". The analysis of a 96-well plate is represented in Fig.3. A positive hit was defined as a chemical compound producing a significant inhibition of the BRET signal. To avoid false positives, standard deviation obtained on 96 wells can be used to set a threshold: for example,

in our experiments, a decrease of 25% of the BRET value was considered as an inhibited signal: this value was willingly higher than 3x standard deviation of control samples. A special attention should be paid to those molecules that might interfere with the absorption properties of the BRET-based assay (fluorescent, colored compounds…). To distinguish between false positive hits or bona-fide specific inhibitors, these compounds should be tested against a BRET signal produced from an unrelated PPI. In case a hit molecule is identified, it should be confirmed by a new BRET measurement performed in triplicate, and also tested on the control strain expressing only the energy donor, to confirm that it does not interfere with the luciferases or the luciferins (substrates) (see details in Suppl. Data). This homogeneous assay (no wash steps are required) can be easily adapted to perform high-throughput screening of large libraries of chemical compounds. With this protocol up to 800 compounds can be manually screened per day.

In this article, we used Nutlin-3 and Nutlin-3a, known inhibitors of the HDM2/p53 interaction, to validate the yeast BRET-based assay as a robust method to identify P2I2s. Indeed, as proof of concept, the cell-based screening assay described here has permitted the discovery, among more than 5,000 compounds tested, of first-in-class chemical structures that can inhibit the CDK5/p25 interaction involved in various human diseases, and notably in Alzheimer's disease[8,13]. Among these inhibitors, tamoxifen was discovered and additional studies have demonstrated that treatment of neuronal cells with this compound affects Tau phosphorylation, a substrate of CDK5[13]. Contrary to these previous research articles, we comprehensively describe here all steps of the protocol (including putative troubleshootings), the statistical Z'-factor (adapted to BRET in yeast, the Z-yBRET factor) and finally the possibility to design alternative versions of the screening assay

by modifying the donors. In this study, we have improved this yeast BRET protocol by using the high-efficiency small donor luciferase NLuc, rather than the RLuc used previously. This results in higher protein expression and signal intensity/stability, and represents a significant improvement when dealing with poorly expressed heterologous proteins. New donor/acceptor couples are still being discovered and optimized, and their use may further enhance the BRET signals produced in our yeast PPI assay. Important advances have been made with the Nano-lanterns which are direct donor-acceptor fusion proteins optimized for BRET-based high bioluminescence[15]. New RLuc8 mutants fused to mutated acceptors such as mVenus, mTurquoise and mKusabiraOrange2, lead to BRET signal more than 8000 mBRET[15].

The protocol described herein associated with new advances in BRET couples should contribute to the discovery of new promising therapeutic candidates that inhibit a variety of PPIs.

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Conflict of Interest

The authors declare no commercial or financial conflict of interest.

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Figure legends

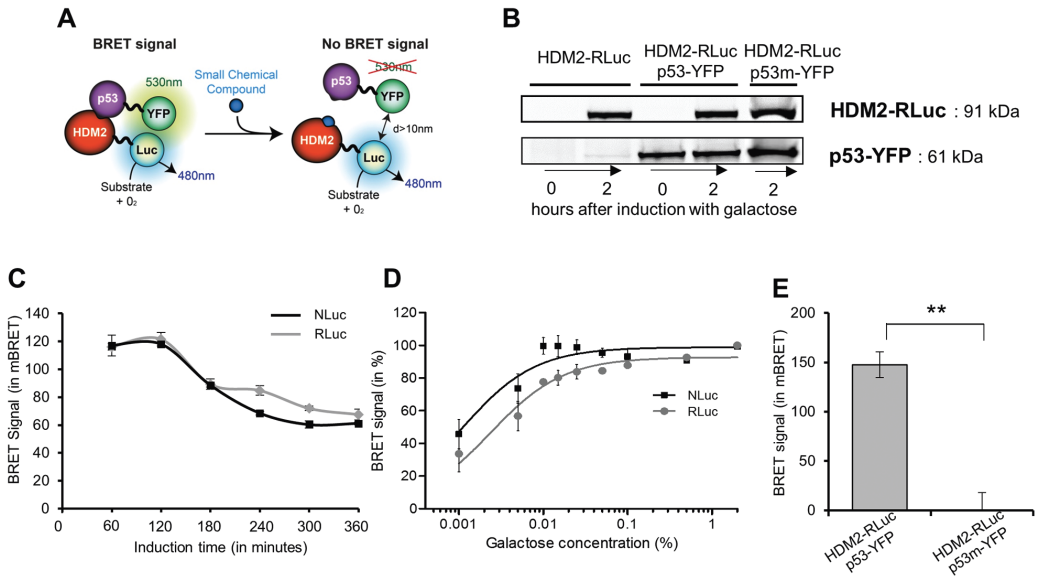


Figure 1. Yeast BRET technique validation and optimization. A. Schematic representation of BRET-based assay, used to detect the interaction between two proteins (*e.g.* HDM2 and p53). **B.** Protein expressions are analyzed by SDS-PAGE followed by Western blotting using antibodies directed against YFP and RLuc, from 25µg of crude extracts produced from the control strain expressing HDM2-RLuc only, and from the test strain expressing HDM2-RLuc and p53-YFP or p53m-YFP. p53m: F19A p53 mutant (F19 residue is involved in the interaction). **C.** Evolution of the BRET signal following induction time. The yeast strains observed express p53-YFP and HDM2-RLuc or HDM2-NLuc (mean ± SD, error bars represent SD, n=3) **D.** Comparison between RLuc and NLuc signals, measured after 2h of induction, with various galactose concentrations. mBRET signals are expressed in % with respect to RLuc signal at 2% of galactose (Error bars represent %RSD, n=3). **E.** Validation of BRET signal specificity. Note the total absence of BRET signal when HDM2-RLuc and p53m-YFP are coexpressed (mean ± SD, error bars represent SD, n=3; **values are significantly different ($p < 0.01$)).

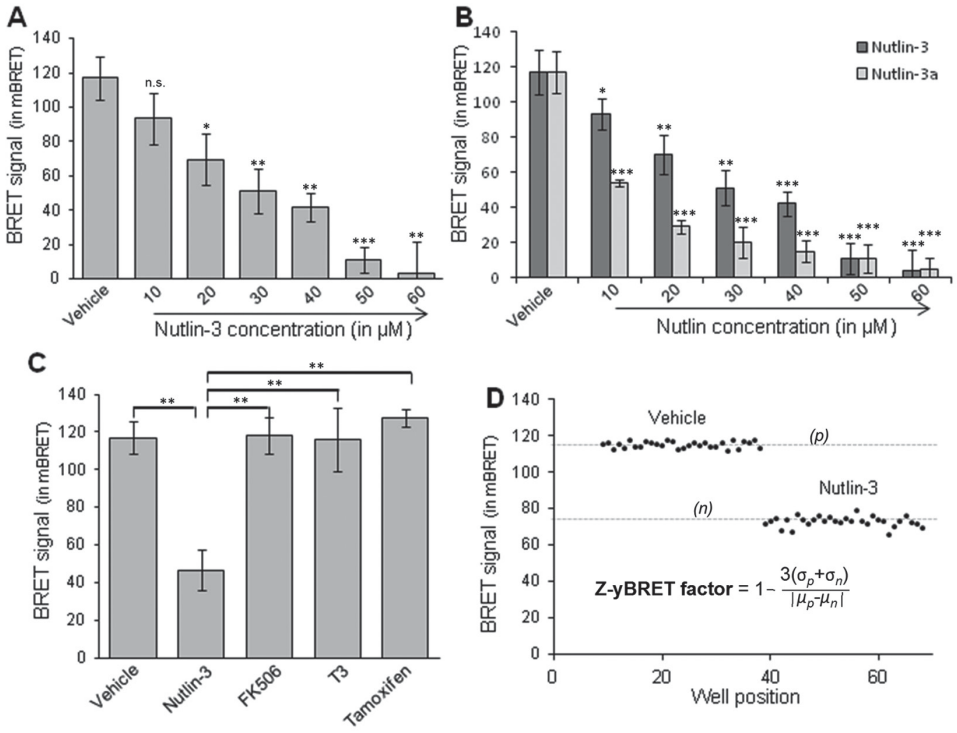


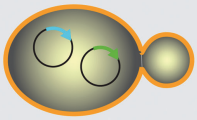
Figure 2. Monitoring the effect of a P2I2 using BRET in yeast. A. Verification of the dose-dependent inhibition of BRET signal by Nutlin-3, a known inhibitor of the HDM2/p53 interaction. RLuc was used as donor protein (mean ± SD, error bars represent SD, n=3). Statistical analyses were done using Student's *t* test. Significance levels are *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$; n.s.: not significant. **B.** Comparison of dose-dependent inhibition of Nutlin-3 and its enantiomer Nutlin-3a using NLuc as donor protein (mean ± SD, error bars represent SD, n=3). Significance levels are *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$. **C.** Specific inhibition of the BRET signal with Nutlin-3 (40µM) compared to unrelated compounds without activity on HDM2/p53 interaction (mean ± SD, error bars represent SD, n=3; **values are significantly different ($p < 0.01$)). **D.** Measurements of the BRET signal in a 96-well plate: 30 wells are filled with the vehicle and 30 others wells are filled with Nutlin-3 (at 20µM). The formula reported in the figure was used to calculate a Z-yBRET factor of 0.67.

Donor vector “Luc”: inducible expression of protein A-Luc fusion

Empty Acceptor vector

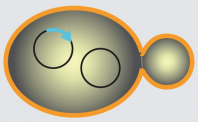
Acceptor vector “YFP”: constitutive expression of protein B-YFP fusion

BRET Strain



Constitutive B-YFP
Inducible A-Luc

Control Strain

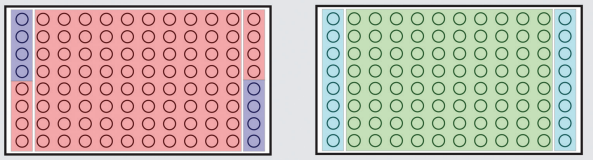


(no acceptor expression)
Inducible A-Luc

Day 1: Over-night culture of yeast cells in SR (Raffinose) - Ura -Leu media at 29°C until A_{600nm} =1.0

Day 2: a] **Preparation of the 96-well plates:**

Distribution of : 1) yeast cells 2) Chemical compounds and DMSO



Control Strain
BRET Strain
DMSO
Tested Molecules in DMSO

b] **Induction of Donor expression:**
=> Galactose addition x h at 29°C

c] **BRET Reading**
1) Substrate (of Luc) addition in each well
2) Plate reading

$$\text{BRET ratio} = \frac{\text{light emitted at acceptor } \lambda}{\text{light emitted at donor } \lambda}$$

BRET Strain => BRET ratio
Control Strain => Background BRET ratio

BRET (mBRET) = (BRETratio - Background BRET ratio) x 1000

Identification of BRET Modulators

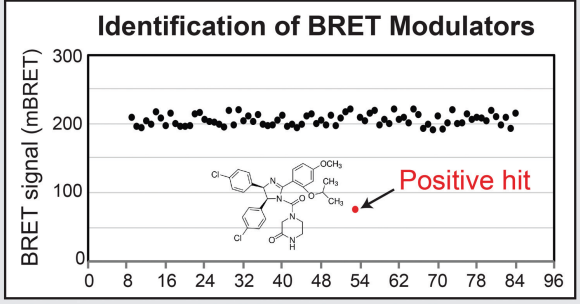


Figure 3. Workflow of the proposed BRET-based screening assay in yeast. In the first (A1 to H1) and in the 12th column (A12 to H12) of each 96-well plate, strains expressing the protein A-Luc donor fusion protein alone (Control strain, A1:D1 and E12:H12) and both donor and acceptor fusion proteins (BRET strain, E1:H1 and A12:D12) are treated with DMSO as controls. The other 80 wells are dedicated to the screening of 80 compounds. Luc: RLuc or NLuc.

Table legend

Table 1. List of the yeast plasmids used in this study.

Table 1

Expressed Protein	Backbone	Marker	Origin	Promoter
HDM2-RLuc	p415GAL1	*LEU2*	CEN/ARS	*GAL1*
HDM2-NLuc	p415GAL1	*LEU2*	CEN/ARS	*GAL1*
p53-YFP	p416GPD	*URA3*	CEN/ARS	*GPD*
p53m-YFP	p416GPD	*URA3*	CEN/ARS	*GPD*

Supplementary material

Cloning strategies

Human HDM2 was amplified by PCR using oSB22 and oSB23 primers and a YFP-HDM2 containing plasmid as a template (kindly provided by Dr. R. Fahraeus and described in Mazars *et al*., 2010). The PCR product was inserted in frame with RLuc in pcDNA3-RLuc vector (Couturier *et al*., 2003) using *Nhe*I/*Age*I restriction enzyme digestion and DNA ligation. The vector obtained, pcDNA3-HDM2-RLuc, was then used as a template for the amplification of HDM2-RLuc cDNA using oSB22 and oSB34 primers. The PCR product was digested with *Bam*HI/*Xho*I enzymes and subsequently cloned in p415-GAL vector to obtain the p415GAL-HDM2-RLuc vector (described in Mumberg *et al*., 1995).

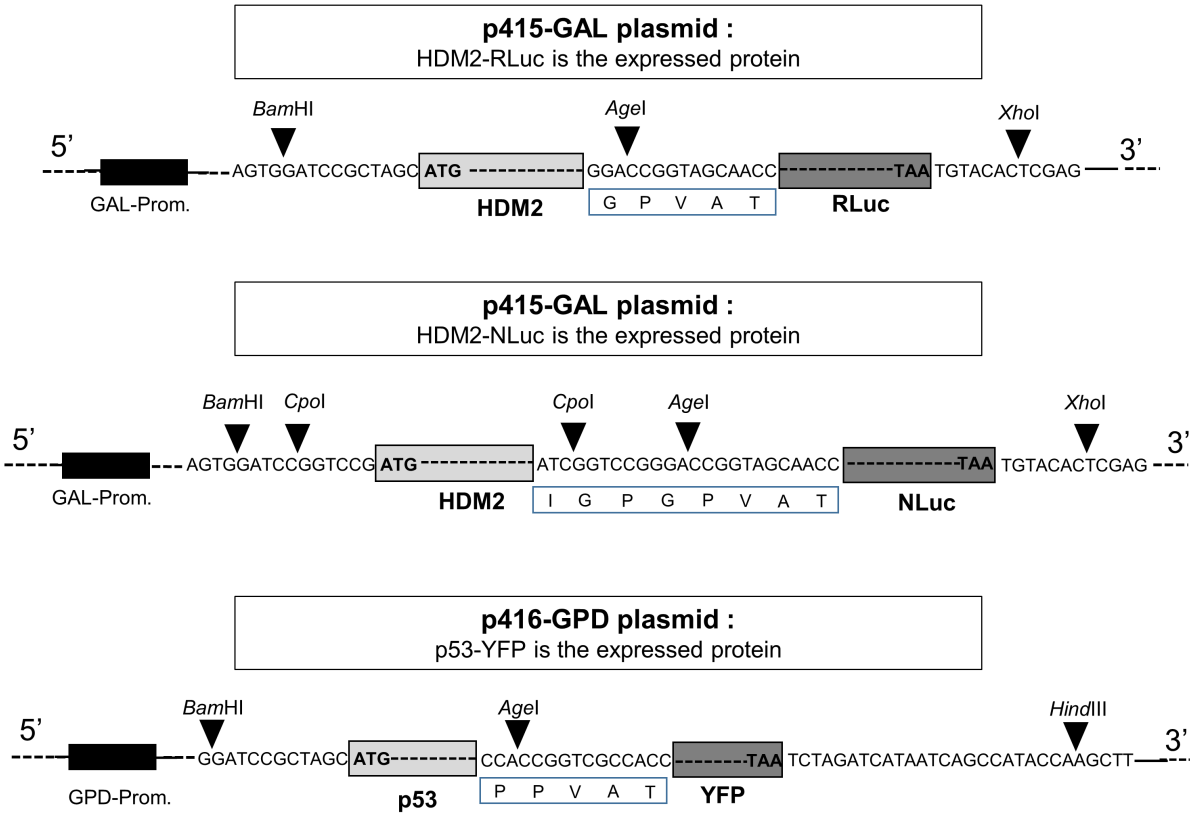
A PCR overlap extension strategy was used to clone HDM2-NLuc in p415-GAL. NLuc (NanoLuc®, Promega) was amplified from pNL.3.2.NF-kB-RE vector (Promega) using NLuc Fw and NLuc Re primers; HDM2 was amplified from p415GAL-HDM2-RLuc vector using HDM2 Fw and HDM2 Re primers. These PCR products were then used as templates for a fusion PCR using NLuc Fw and HDM2 Re primers, *Bam*HI/*Xho*I digested and cloned in p415-GAL to obtain p415GAL-HDM2-NLuc vector.

Human CDK5 was amplified by RT-PCR using NheI-hCDK5_Sens and hCDK5-AgeI_AS primers and cloned in frame with YFP in the pcDNA3-YFP vector (described in Couturier *et al*., 2003), using *Nhe*I/*Age*I restriction enzyme digestion and DNA ligation. NheI-CDK5-YFP-HindIII PCR product was generated using NheI-hCDK5_Sens and pcDNA3-HindIII_AS primers and vector pcDNA3-hCDK5-YFP as a template. This PCR product and p416-GPD vector (described in Mumberg *et al*., 1995) were then digested with *Nhe*I/*Hin*dIII restriction enzymes and ligated together to gain the p416GPD-hCDK5-YFP expressing vector.

Human p53 and a human p53 mutant (F19A) were amplified using oSB28 and oSB29 primers and with RLuc-p53 and RLuc-p53.F19A containing plasmids as templates (kindly provided by Dr. R. Fahraeus and described in Mazars *et al*., 2010). PCR products were then digested by *Bam*HI/*Age*I and inserted in frame with YFP in p416GPD-CDK5-YFP digested with *Bam*HI/*Age*I.

Primer name **(*restriction enzyme*)**	**Sequence (5'-…-3')**
oSB22 Fw ***(Bam*HI/*Nhe*I)**	CTCT***GGATCC*GCTAGC**ATGTGCAATACCAACATGTCTG
oSB23 Re	CT***ACCGGT***CCGGGGAAATAAGTTAGCAC

(AgeI)	
oSB34 Re ***(XhoI/BsrGI)***	CGC**CTCGAG*TGTACA***TTACTGCTCGTTCTTCAGCAC
NLuc Fw **(*CpoI*/*AgeI*)**	CCCAT**CGGTCC**GGG**ACCGGT**AGCAACCATGGTCTTCACACTCGAAGATTT
NLuc Re **(*XhoI*)**	AATTATTTTA**CTCGAG**TCACGCCAGAATGCGTTCGCA
HDM2 Fw **(*Bam*HI/*CpoI*)**	TAAATATAAA***GGATCC*GGTCCG**ATGTGCAATACCAACATGTCTG
HDM2 Re **(*AgeI*/*CpoI*)**	GCT**ACCGGT**CC**CGGACCG**ATGGGGAAATAAGTTAGCACAATCA
NheI-hCDK5_Sens **(*NheI*)**	ACCCAA**GCTAGC**ATGCAGAAATACGAGAAAC
hCDK5-AgeI_AS **(*AgeI*)**	GTGGCG**ACCGGT**GGACAGAAGTCGGAGAAG
pcDNA3-HindIII_AS **(*Hin*dIII)**	ACCTCTAC**AAGCTT**GGTATGGCTGATTATG
oSB28 Fw ***(Bam*HI/*NheI*)**	CTCT***GGATCC*GCTAGC**ATGGAGGAGCCGCAGTCAGATCC
oSB29 Re ***(AgeI)***	CT***ACCGGT***GGGTCTGAGTCAGGCCCTTCTG



Supplementary figure: Simplified map of the different fusion proteins used in this study.

Step by step protocol and troubleshootings

1/ Preculture preparation • TIMING ~15 min

The day before the BRET measurement, the two yeast strains (the one expressing the BRET donor only and the second expressing both BRET donor and acceptor) are grown over-night at 29°C in SR-Ura-Leu (SR for Synthetic Raffinose medium). These preparations constitute two distinct precultures.

2/ 96-well plate preparation • TIMING ~20 min

(i) The following day, the OD_{600nm} are measured. The OD_{600nm} obtained are often higher than 1. Dilute these precultures to an optimal OD_{600nm} equal to 1.

Δ CRITICAL STEP: These dilutions still occur in the media containing Raffinose.

? TROUBLESHOOTING

- In case the OD_{600nm} of the overnight culture is too different from 1:

- Starting from 10-20 picked colonies, a 18-24 hours pre-inoculum can be subsequently diluted to OD_{600nm} equal to 0.04 and grown for 15-18 hours at 29°C.

(ii) Fill the 96-well plate (#655083, Greiner Bio-one) with 36µL of yeast culture using a multichannel pipette.

Δ CRITICAL STEP: The first and the 12^{th} lines of the 96-wells plate are dedicated to controls. 8 wells are filled by the yeast strain expressing the BRET donor only (Fig. 3).

(iii) Add 0.4µL of the chemical compounds to perform a triplicate, at the desired final concentration.

Δ CRITICAL STEP 1: Verify in advance that the chemical compound to test does not precipitate in the yeast growth media.

Δ CRITICAL STEP 2: The final concentration in DMSO should not exceed 3%.

(iv) Add 3.6µL of Galactose 20% to induce the expression of the BRET donor.

(v) Position the plate in a vibrating shaking platform for 2 hours at 29°C.

Δ CRITICAL STEP 1: The induction time and galactose concentration should be optimized considering the protein-protein interaction studied.

Δ CRITICAL STEP 2: To prevent media evaporation during this induction, the plate could be sealed by a suitable lid or parafilm and the vibrating shaking platform should be placed in a 29°C incubator.

3/ BRET measure • TIMING ~20 min

(i) Prepare a fresh 25µM dilution of coelenterazine h (37.5µL of the stock solution at 1mM are diluted in 1500µL PBS). If using NLuc, the Nano-Glo® Luciferase Assay substrate is first diluted 1000 times in PBS (1.5µL in 1500µL PBS).

(ii) 10µL of the freshly diluted substrate are added in each well tested. The final concentration of coelenterazine h is 5µM while the Nano-Glo® Luciferase Assay substrate is diluted 5000 times respect to stock solution.

Δ CRITICAL STEP: Coelenterazine h should be kept as much as possible protected from light.

(iii) Load the multiwell plate in the BRET reader.

(iv) Choose the appropriate filters to measure consecutively, for each well, the donor and acceptor emissions. The same plate can be measured repeatedly to ensure a proper reading window in which luciferase activity is decreasing.

Δ CRITICAL STEP: Be sure to avoid measurement of the donor emission on the overall plate and, then, the same for the acceptor emission as both these measure must be performed in a short time interval on the same well.

4/ Data processing • TIMING ~15 min

(i) Calculate the BRET ratio for all the wells containing the yeast co-expressing the donor and the acceptor as: acceptor emission/ donor emission and correct it with the Background BRET ratio obtained for the yeast expressing the donor only as: BRET signal (mBRET) = (BRET ratio – Background BRET ratio) x1000.

Δ CRITICAL STEP: Verify that the emission values obtained are higher than 100 for RLuc, 5000 for NLuc.

? TROUBLESHOOTING

- In case of the emission values obtained are too low:

- check your substrate stock solution and if necessary, prepare a fresh one;

- Raffinose solution must be sterilized by filtering and stored at 4°C for up to one month. Raffinose may easily hydrolyze in galactose, glucose and fructose. Glucose may repress donor fusion protein expression.

- verify the expression of your proteins of interest by western blot for example, after protein extraction;

- the chemical compounds tested are too concentrated.

- In case of the BRET signals are too low:

- verify the protein-protein interaction by co-immunoprecipitation;

- check all possible N- or C-terminal orientations of the donor and acceptor groups;

- if working with truncated protein versions (single domains responsible of interaction), add signal peptides for correct subcellular localization (*e.g*. nuclear localization signal for nuclear proteins);

- If the emission values are perturbed in some wells, the corresponding chemical compound is probably toxic for the yeast and its effect cannot be observed by this technique.

(**ii**) A hit molecule is defined as a chemical compound presenting a signal inhibited by 25% in comparison with the vehicle treatment.

(**iii**) In case of a hit molecule is identified, this should be confirmed by new BRET measurements where the chemical compound will be added on the yeast expressing only the donor of energy. To distinguish between a false positive hit molecule or bona-fide specific inhibitor, the compound should be tested against a BRET signal produced from an unrelated protein-protein interaction.

Δ CRITICAL STEP: this experiment will confirm that the chemical compound does not disrupt the BRET signal and is a true protein-protein interaction inhibitor, *i.e*. that it does not interfere

with the *Renilla* Luciferase or with Coelenterazine h. Also, effects of colored or fluorescent compounds should be particularly verified as they can modify the light transmission properties of the medium (Couturier and Deprez, 2012).

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