Bioluminescence Resonance Energy Transfer 2 (BRET2)-Based RAS Biosensors to Characterize RAS Inhibitors

INTRODUCTION

While the current in vitro techniques for monitoring RAS-effector inhibition by small molecules or macromolecules are well established and widely used [e.g., AlphaScreen, HTRF (Upadhyaya et al., 2015; Waldmann et al., 2004; Wu et al., 2013)], the cell-based techniques are limited to the immuno-precipitation of RAS with its partners and to the analysis of the phosphorylation status of kinases belonging to RAS dependent signaling pathways (Upadhyaya et al., 2015; Welsch et al., 2017). The latter method is an indirect measure of RAS inhibition by the compounds whilst the immuno-precipitation is a labor-intensive method that is not compatible with testing compounds or macromolecules at a large scale. Therefore, a cell-based assay allowing a large-scale test of inhibitors would be valuable, as it would give information on the behavior of the inhibitors in the cellular environment. Accordingly, we have engineered genetically encoded Bioluminescence Resonance Energy Transfer 2 (BRET2)-based RAS biosensors (Bery et al., 2018). These biosensors are optimized and comprised a full-length RAS donor molecule (wild-type and mutant K, N, and HRAS), to respect its intracellular localization, and several different known acceptor partners like PI3Kα, PI3Kγ and CRAF RAS binding domains (RBD) and the RAS associating (RA) domain of RALGDS. We also used as acceptor the full-length CRAF and PI3Kα effectors to study their respective downstream signaling pathways. Therefore, using this BRET-based RAS biosensors toolbox, we characterized the cellular properties of various RAS inhibitors comprising anti-RAS Design Ankyrin Repeat Proteins (DARPins) (Guillard et al., 2017) and anti-RAS intracellular domain antibody (iDAb RAS) (Bery et al., 2018) macromolecules and antibody derived anti-RAS compounds (Bery et al., 2018; Quevedo et al., 2018). Here we describe a set of protocols that include step-by-step instructions to monitor PPIs including macromolecules (Basic Protocol 2) and small molecules (Basic Protocol 3).We also describe a protocol to detect the effect of RAS inhibitors on the RAS downstream pathways by immunoblot (Basic Protocol 4) and finally a protocol explaining how to calculate the BRET ratio obtained in the previous protocols (Basic Protocol 5).

BASIC PROTOCOL 1

BRET2 DONOR SATURATION ASSAY WITH RAS BIOSENSORS

The BRET donor saturation assay is performed to determine whether two proteins interact specifically together (Mercier, Salahpour, Angers, Breit, & Bouvier, 2002). A fixed amount of donor plasmid is transfected in cells with an increased amount of acceptor plasmid. If the proteins interact, the BRET signal will increase and reach saturation while if they do not interact, the BRET signal will increase linearly (Fig. 1A, B). An outline of the stages involved in BRET assays is shown in Figure 2A, B, and the specific example of RAS family biosensors in Figure 2C.

Materials

HEK293T cells (ATCC# CRL-3216, RRID:CVCL_0063)
Penicillin/streptomycin (Thermo Fisher, cat. no. 10378016)
RLuc8 construct (see Table 1)
GFP2 construct (see Table 1)
OptiMEM medium (Thermo Fisher, cat. no. 31985070)
Lipofectamine 2000 (Thermo Fisher, cat. no. 11668019)
Trypsin (Thermo Fisher, cat. no. 25300054)
DMEM (Thermo Fisher, cat. no.10566016)

Fetal bovine serum (FBS; Sigma)
Phosphate-buffered saline (PBS; Thermo Fisher, cat. no. 10010023)
OptiMEM no red phenol (Thermo Fisher, cat. no. 11058021)
Coelenterazine 400a (Cayman Chemicals, cat. no. 16157)
100% Ethanol (EtOH)
RAS biosensors plasmids [(Bery et al., 2018); see Table 1]
pEF-myc-cyto, empty plasmid (Invitrogen, cat. no. V89120)
6-well plates (Corning)
37°C incubator
15-ml Falcon tubes
Centrifuge
White 96-well plates, clear bottom with white tapes (PerkinElmer, cat. no. 6005181)
Envision instrument 2103 Multilabel Reader (PerkinElmer, cat. no. 2103)
White tape (PerkinElmer)
SpeedVac instrument
Multichannel pipettes
BRET2 Dual Emission optical module: 515 nm - 30 and 410 nm - 80 (PerkinElmer, cat. no. 2100–8140)

Day 1: Cell seeding

1. Plate 650,000 HEK293T in each well of a 6-well plate in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin. One well corresponds to one point of the titration curve. Two plates are needed for one titration curve. Cells need to be no more than 70% to 80% confluent before being plated in a 6-well plate in order to get optimal cell density on Day 2 for transfection.

Day 2: Cell transfection

2. Mix together a fixed amount of RLuc8 construct (typically 50 ng) and a varying amount of GFP2 construct (see quantity in Table 2). The pEF-myc-cyto empty plasmid is used to equalize total amount of transfected DNA between wells. The total DNA amount transfected per well is 1.6 µg. Note that the 1:0.25 and 1:0.5 ratios are not always performed (only when needed for strong interactions).
3. Prepare the DNA mix from step 2 in 100 µl OptiMEM medium.
4. Add 4 µl Lipofectamine 2000 transfection reagent in a separate tube with 100 µl OptiMEM medium.
5. Add the DNA mix from step 3 into the Lipofectamine mix from step 4, mix and leave for 20 min at room temperature (RT is 25°C).
6. Add 200 µl of transfection mix into each well and incubate the cells for 24 hr at 37°C.

Day 3: BRET plate preparation

7. Add 500 µl trypsin per well (2 min at RT) to detached cells and neutralize the trypsin by adding 1 ml of complete DMEM (with 10% FBS).
8. Count the cells of only one or two conditions per BRET pair, i.e., one RLuc8 donor construct + one GFP2 acceptor construct such as KRASG12D/CRAF RBD (assuming that the cell number is similar between all the wells after checking under the microscope).
9. In a 15-ml Falcon tube, add 1 ml PBS and add 500,000 cells. Centrifuge 5 min at 240 Å~ g, 20°C, aspirate the supernatant, and add 1 ml OptiMEM no phenol red medium + 4% FBS. Carefully resuspend the cells.
10. Seed 100 µl of cells per well of a white clear bottom 96-well plate. Make quadruplicates for each point. Leave the cells for an additional 20 to 24 hr at 37°C before the BRET reading.

Day 4: GFP2 measurement

11. Read theGFP2 fluorescence with excitation and emission peaks set at 405nmand 515 nm, respectively, on an Envision instrument (2103 Multilabel Reader, PerkinElmer).

12. After the GFP2 reading, tape the clear bottom 96-well plate with a white tape (PerkinElmer) for BRET2 and RLuc8 readings.

Day 4: Prepare BRET substrate (Coelenterazine 400a)

13. Resuspend 1 mg of Coelenterazine 400a in 10.2 ml of 100% EtOH to make a 250 µM solution.

14. Divide this solution into 20 aliquots of 500 µl.

15. Evaporate the ethanol from the solution with a SpeedVac instrument and store aliquots at −80°C (stable up to 1 year at −80°C when dry). One aliquot is enough to read one 96-well plate. Coelenterazine 400a substrate is light sensitive, protect from light with aluminum foil.

Day 4: BRET measurement

16. Resuspend an aliquot of BRET substrate Coelenterazine 400a in 500 µl of 100% EtOH.

17. Add 750 µl OptiMEM no phenol red + 4% FBS in this resuspended aliquot. This makes 1.25 ml of BRET substrate at 100 µM.

18. Add 10 µl of substrate per well of the 96-well plate with a multichannel pipette (10 µM final concentration).

19. BRET2 signal is read directly after addition of substrate on the cells, using an Envision instrument with the BRET2 Dual Emission optical module (515 nm – 30 and 410 nm – 80, PerkinElmer). Read the BRET column by column (add the substrate on column 1, carry out the BRET reading; add the substrate on column 2, carry out the BRET reading etc.).

If a machine with BRET2 capabilities and with injectors is available (e.g., CLARIOstar or PHERAstar (BMG Labtech)), the plate can be read in a well mode where the substrate is injected in well A1, BRET read on A1, substrate injected on A2, BRET read on A2, . . . More generally, BRET2 can be detected by microplate reader (the most popular) or scanning spectrometer (Pfleger&Eidne, 2006). The microplate reader must have the capacity to sequentially or simultaneously detect filtered light within two distinct wavelength windows (410 nm – 80 for the donor and 515 nm – 30 for the acceptor) in one microplate well before moving to the next one, when using coelenterazine 400a as substrate, RLuc8 as donor and GFP2 as acceptor. Suitable microplate readers include, but are not limited to, the Envision or VICTOR (PerkinElmer), the Mithras LB 940 (Berthold Technologies), the FLUOstar Optima or POLARstar Optima or CLARIOstar or PHERAstar (BMG Labtech).

20. Measure total RLuc8 luminescence with the Luminescence 400 to 700-nm wavelength filter.

BASIC PROTOCOL 2

BRET2 COMPETITION ASSAY WITH anti-RAS MACROMOLECULES

The donor saturation assay described in Basic Protocol 1 is a mandatory step to determine the right donor/acceptor ratio to express within the cells in order to avoid the titration of inhibitors in a competition assay. Indeed, the acceptor molecule has to be expressed to a higher level compared to the donor in order to maximize the BRET signal (Couturier & Deprez, 2012). However, excess of acceptor but also of both acceptor/donor pair (excess of the monitored complex) might titer a potential active molecule (or macromolecule) leading to its inability to promote the expected decrease in signal. Therefore, in order to monitor such a decrease with a macromolecule or a compound, the correct quantity of donor and

the donor/acceptor ratio must be determined for each interaction tested with a donor titration curve. We have accomplished this work and determined the right ratio for each biosensor. Hence, our biosensors are ready to select inhibitors of RASeffector interactions (Fig. 3A). Using these competition assays, we successfully characterized an anti-RAS intracellular domain antibody (iDAb RAS) (Bery et al., 2018) and anti-RAS Design Ankyrin Repeat Proteins (DARPins) (Guillard et al., 2017) overexpressed within the cells. The range of macromolecules should not be limited to the ones we used, as any anti-RAS macromolecules of choice could be tested in these competition assays.

Additional Materials (also see Basic Protocol 1)
RAS competitors' plasmids [see (Bery et al., 2018; Guillard et al., 2017) and see Table 3]
Day 1: Cell plating
1. Plate 650,000 HEK293T in each well of a 6-well plate in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin.

Day 2: Cell transfection
2. Mix together a fixed amount of RLuc8 construct (typically 50 ng) and GFP2 construct (see quantity in Table 4) with a varying amount of pEF-iDAb or DARPin competitor (100, 500 and 1000 ng). Always transfect a negative (i.e., non-RAS binder) and a positive control (i.e., iDAb RAS) in a competition assay. The pEF-myc-cyto empty plasmid is used to equalize total amount of transfected DNA between wells. The total DNA amount transfected per well is 1.6 µg. Increased competitor points could be achieved (instead of 3 as indicated) to get a dose response curve if needed. This would be valuable to compare different inhibitors efficiency.
3. Prepare the DNA mix from step 2 in 100 µl OptiMEM medium.
4. Add 4 µl Lipofectamine 2000 transfection reagent in a separate tube with 100 µl OptiMEM medium.
5. Add the DNA mix from step 3 in the Lipofectamine mix from step 4, mix, and wait for 20 min at room temperature.
6. Add 200 µl transfection mix in each well and incubate the cells for 24 hr at 37°C.

Day 3
7. Complete cell seeding in a 96-well plate, as described in Basic Protocol 1 (see steps 7 to 10).

Day 4
8. Perform BRET measurement, as described in Basic Protocol 1 (see steps 11 to 12 and 16 to 20).

ALTERNATE PROTOCOL 1
BRET2 COMPETITION ASSAY WITH anti-RAS MACROMOLECULES WITH EGF STIMULATION OF TARGET CELLS
The protocol described below is used to characterize the inhibitory potency of anti-RAS macromolecules against wild-type RAS (RLuc8-K, N and HRASWT) and its effectors (e.g., GFP2-CRAFFL).

Day 1 and Day 2 of this protocol are identical to the Basic Protocol
2. Modifications are made from Day 3. Additional Materials (also see Basic Protocols 1 and 2) Epidermal Growth Factor, EGF (Life Technologies, cat. no. PHG0311)

Day 3: BRET plate preparation
1. Add 500 µl trypsin per well (2 min at room temperature) to detached cells and neutralize

the trypsin by adding 1 ml complete DMEM (with 10% FBS).
2. Count the cells of only one or two conditions (see Basic Protocol 1, step 8).
3. In a 15-ml Falcon tube, add 1 ml PBS and add 500,000 cells. Centrifuge 5 min at 240 Å~ g, 20°C, aspirate the supernatant, and add 1 ml OptiMEM no phenol red medium + 1% FBS. Resuspend the cells carefully.
4. Seed 90 µl of cells (_45,000 cells) per well of a white clear bottom 96-well plate. Make quadruplicates for each point. Incubate the cells for 24 hr at 37°C before EGF stimulation and BRET measurement.

Day 4: EGF treatment
5. Add 10 µl OptiMEM no phenol red + 1% FBS into the non-treated EGF rows (rows A-D).
6. Add 10 µl of a 500 ng/ml EGF (Life Technologies) in OptiMEM no phenol red + 1% FBS per row (rows E-H) with a multichannel pipette (see Table 5).
7. Incubate 5 min at 37°C and proceed to the BRET reading.

Day 4: BRET measurement
8. Perform the BRET measurement as in the Basic Protocol 1 (steps 11 to 12 and 16 to 20), except that the BRET reading is done row-by-row and not column-by-column. Start the BRET reading with the EGF treated rows (rows E-H, add the substrate on row E, carry out the BRET reading; add the substrate on row F, carry out the BRET reading etc.). Finish with the non-treated rows.

BASIC PROTOCOL 3
BRET2 COMPETITION ASSAY WITH anti-RAS SMALL MOLECULES
This protocol should be used with anti-RAS compounds or small molecules. It will give insight into the inhibitory behavior of the compounds against the interaction between
mutant RAS (K, N, and HRAS) and their different effectors. Below is a detailed protocol for performing a competition assay with one BRET RAS biosensor (e.g.,KRASG12D/CRAFFL) in a full 96-well plate. It can be scaled up depending on the number of compounds and RAS PPI to be tested. Additional Materials (also see Basic Protocol 1) Compounds at 10 mM in 100% dimethyl sulfoxide (DMSO; Sigma, cat. no. D2650-100ML)

Day 1: Cell seeding
1. Plate 650,000 HEK293T cells in each well of a 6-well plate as done for Basic Protocol 2 (step 1).

Day 2: Cell transfection
2. Mix together a fixed amount of RLuc8 construct (typically 50 ng) andGFP2 construct (see quantity in Table 6). Four transfected wells of a 6-well plate typically yield enough cells to perform a competition assay in a full 96-well plate.
3. Prepare the DNA mix from step 2 in 100 µl OptiMEM medium.
4. Add 4 µl Lipofectamine 2000 transfection reagent in a separate tube with 100 µl OptiMEM medium.
5. Add the DNA mix from step 3 in the Lipofectamine mix from step 4, mix, and wait for 20 min at room temperature.
6. Add 200 µl transfection mix in each well and incubate the cells for 24 hr at 37°C.

Day 3: BRET plate preparation
7. Add 500 µl trypsin per well (2 min at room temperature) to detached cells and neutralize the trypsin by adding 1 ml complete DMEM (with 10% FBS).
8. Pool the cells in a 15-ml Falcon tube and count the cells with a hemacytometer or any

other cell counting method.
9. For each BRET RAS biosensor, in a new 15-ml Falcon tube, add 5 ml PBS and add 5 million cells. Centrifuge 5 min at 240 Å~ g, 20°C, aspirate the supernatant and add 12 ml of OptiMEM no phenol red medium + 4% FBS. Resuspend the cells carefully.
10. Seed 90 µl of cells (_38,000 cells) per well of a white clear bottom 96-well plate. Leave the cells for 4 hr at 37°C before adding the compounds.

Day 3: Compounds preparation
11. Dilute 10 mM stock of the compounds in 100% DMSO in OptiMEM no red phenol + 4% FBS, in order to reach 10Å~ (the final concentration; 2% DMSO for each concentration). The final concentrations in the cells are 0, 5, 10, and 20 µM; therefore, the intermediate 10Å~ concentrations are 0, 50, 100, and 200 µM (see Table 7).
12. Add 10 µl of 10Å~ compounds in each well of the 96-well plate in order to make 0, 5, 10, and 20 µM as final concentrations with 0.2% DMSO each. Perform quadruplicates for each point. For all compounds, one plate column is used for the DMSO control and three other columns for the compound itself (Table 8).
13. Incubate the cells for an additional 20 hr at 37°C before the BRET reading.

Day 4
14. Perform BRET measurement, as described in the Basic Protocol 1 (see steps 11 to 12 and 16 to 20).

ALTERNATE PROTOCOL 2
BRET2 COMPETITION ASSAY WITH anti-RAS SMALL MOLECULES WITH EGF STIMULATION OF TARGET CELLS – 20H INCUBATION
This follows the same procedure as Basic Protocol 3 except with the following modifications on Day 3 and Day 4. It applies only when wild-type RAS is used as a donor (RLuc8-RASWT) and a RAS effector used as acceptor (e.g., GFP2-CRAFFL). This protocol should be used to determine whether a small molecule/compound is active against wild-type RAS PPI (Fig. 3B). Additional Materials (also see Basic Protocols 1 and 3) Epidermal Growth Factor (EGF; Life Technologies, cat. no. PHG0311)

Day 3: BRET plate preparation
1. Add 500 µl trypsin per well (2 min at room temperature) to the detached cells and neutralize the trypsin by adding 1 ml complete DMEM (with 10% FBS).
2. Pool the cells in a 15-ml Falcon tube and count the cells with a hemacytometer or any other cell counting method.
3. For each BRET RAS biosensor, in a new 15-ml Falcon tube, add 5 ml PBS and 6 million cells. Centrifuge 5 min at 240 Å~ g, 20°C, aspirate the supernatant, and add 12 ml OptiMEM no phenol red medium + 1% FBS. Resuspend the cells carefully.
4. Seed 81 µl of cells (_41,000 cells) per well of a white clear bottom 96-well plate. Incubate the cells for 4 hr at 37°C before adding the compounds.

Day 3: Compounds preparation
5. Dilute 10 mM stock compounds in 100% DMSO in OptiMEM no red phenol + 1% FBS, in order to reach 10Å~ (the final concentration; using the same compound preparation from step 12 in Basic Protocol 3).
6. Add 9 µl of 10Å~ compounds in each well of the 96-well plate. Make quadruplicates for each point. For each compound, one plate column is dedicated to a DMSO only-control and three other columns for the compound itself (see Table 9).
7. Incubate the cells for an additional 20 hr at 37°C before EGF treatment (day 4) and BRET

reading.

Day 4: EGF treatment
8. Add 10 µl OptiMEM no phenol red + 1% FBS on the non-treated EGF rows (rows A-D).
9. Add 10 µl of a 500 ng/ml EGF in OptiMEM no phenol red + 1% FBS per row (rows E-H) with a multichannel pipette (see Table 9).
10. Incubate 5 min at 37°C and proceed to the BRET reading.
Day 4: BRET measurement
11. Perform the BRET measurement as in Alternate Protocol 1 (step 8).

ALTERNATE PROTOCOL 3
BRET2 COMPETITION ASSAY WITH anti-RAS SMALL MOLECULES
This is the protocol to perform one competition assay (one BRET RAS biosensor) in a full 96-well plate when the small molecules are incubated for a short time (here 3 hr). This should avoid off-target effect of the compounds on the cells. As for the long incubation protocol (20 hr incubation), the cells can be either stimulated by EGF (wild-type RAS) or not (mutant RAS). The following protocol describes both cases. For materials, see Basic Protocols 1 and 3 and Alternate Protocol 2.

Day 1: Cell seeding
1. Plate 650,000 HEK293T cells in each well of a 6-well plate.
Day 2: Cell transfection
2. Mix together a fixed amount of RLuc8 construct (typically 50 ng) andGFP2 construct
(see quantity in Table 10). Four transfected wells of a 6-well plate typically yield enough cells to perform a competition assay in a full 96-well plate.
3. Prepare the DNA mix from step 2 in 100 µl OptiMEM medium.
4. Add 4 µl Lipofectamine 2000 transfection reagent in a separate tube with 100 µl OptiMEM medium.
5. Add the DNA mix from step 3 in the Lipofectamine mix from step 4, mix, and wait for 20 min at room temperature.
6. Add the 200 µl transfection mix in each well and incubate the cells for 24 hr at 37°C.

Day 3: BRET plate preparation
7. Add 500 µl trypsin per well to detach the cells. Neutralize the trypsin by adding 1 ml complete DMEM.
8. Pool the cells in a 15-ml Falcon tube and count the cells with a hemacytometer or any other cell counting method.
9. For each BRET RAS biosensor, in a new 15-ml Falcon tube, add 5 ml PBS and 6 million cells. Centrifuge 5 min at 240 Å~ g, 20°C, aspirate the supernatant, and add 12 ml of OptiMEM no phenol red medium, no FBS. Resuspend the cells carefully.
10. Seed 81 µl of cells (_41,000 cells) per well of a white clear bottom 96-well plate. Incubate the cells for 24 hr at 37°C before adding the compounds.

Day 3: Compound preparation
11. Add 9 µl of 10Å~ compounds in each well of the 96-well plate. Incubate the cells for 3 hr (including or not a 5 min EGF treatment at 50 ng/ml final concentration; see Alternate Protocol 2). The full description of compounds preparation is in Basic Protocol 3 (see steps 11 to 13) and in Alternate Protocol 2 (see steps 5 to 7).

Day 3: BRET measurement
12. See Basic Protocol 3 (no EGF treatment) or Alternate Protocol 1 (EGF treatment) for

details on the BRET reading.

BASIC PROTOCOL 4

IMMUNOBLOT TO ASSESS EFFECT OF RAS INHIBITORS ON THE RAS DOWNSTREAM PATHWAYS

When full-length effectors (PI3KαFL or CRAFFL) are used in a BRET2 competition assay with either small molecules or macromolecules (with or without EGF stimulation), we showed the possibility to observe the effect of these inhibitors on the corresponding RAS downstream pathway by immunoblots. We could analyze the RAS-RAF-MEKERK and RAS-PI3Kα-AKT signaling pathways with RAS/CRAFFL and RAS/PI3KαFL BRET pairs, respectively (Bery et al., 2018). To perform these immunoblots, we used the same transfected cells than the one used for the BRET2 competition assays described previously. Therefore, days 1 and 2 are similar to the other protocols; changes are only made on Day 3 and Day 4. Here, we describe the protocol for immunoblot using cells from Basic Protocols 2 and 3 and Alternate Protocols 1 and 2. Additional Materials (also see Basic Protocols 1, 2, 3 and Alternate Protocols 1 and 2) Cell lysis buffer (see recipe)

Day 3: Immunoblot plate preparation

1. Plate 1.6 million transfected cells, from a BRET2 competition assay (Basic Protocols 2 and 3 and Alternate Protocols 1 and 2), in a 6-well plate (one well per condition) with 1 ml of OptiMEM no phenol red + 4% FBS (or 1% FBS for EGF stimulation).
2. Incubate the cells for 4 hr at 37°C before addition of compounds.

Day 3: Compound preparation

3. Dilute in 1.2 ml OptiMEM no phenol red + 4% FBS (or 1% FBS for EGF stimulation) 10 mM stock of the compounds in 100% DMSO, in order to reach 2Å~ (the final concentration; 0.4% DMSO for each concentration): 10, 20, and 40 µM for 5, 10, and 20 µM final concentrations, respectively.
4. Add 1 ml of 2Å~ compounds in each well of the 6-well plate.
5. Incubate the cells for an additional 20 hr at 37°C.

Day 4: Immunoblot

6. Optional: If EGF stimulation: Treat cells with 50 ng/ml final concentration of EGF for 5 min at 37°C.
7. Lyse the cells in 0.3 ml cell lysis buffer per well of the 6-well plate.
8. Resolve the proteins as described in Bery et al. (2018).

ALTERNATE PROTOCOL 4

BRET2 COMPETITION ASSAY WITH SMALL MOLECULES – WESTERN BLOTS FOR SHORT INCUBATION (3 HOURS) WITH OR WITHOUT EGF TREATMENT

Here we describe the protocol for immunoblot using cells from Alternate Protocol 3 as few parameters change compared to Basic Protocol 4. For materials, see Basic Protocol 4.

Day 3: Immunoblot plate preparation

1. Plate 2 million transfected cells, from a BRET2 competition assay (Alternate Protocols 3), in a 6-well plate (one well per condition) in 1.5 ml OptiMEM no phenol red + 0.5% FBS.
2. Incubate the cells for 24 hr at 37°C before addition of compounds.

Day 3: Compound preparation

3. Dilute in 1.2 ml OptiMEM no phenol red, no FBS, 10 mM stock of the compounds in 100% DMSO, in order to reach 4Å~ (the final concentration; 0.8% DMSO for each concentration): 20, 40, and 80 µM for 5, 10, and 20 µM final concentrations, respectively.

4. Add 0.5 ml of 4Å~ compounds in each well of the 6-well plate.
5. Incubate the cells for an additional 3 hr at 37°C (including or not a 5 min EGF treatment at 50 ng/ml final concentration).
6. Perform cell lysis and immunoblot as described in steps 7 to 8 of Basic Protocol 4.

BASIC PROTOCOL 5
BRET2 CALCULATIONS FOR EACH PROTOCOL
A BRET signal occurs when the donor (RLuc8 construct) and acceptor (GFP2 construct) molecules are in close proximity (_10 nm). BRET ratio calculation for donor saturation assay The BRET signal or BRET ratio corresponds to the light emitted by the GFP2 acceptor constructs (515 nm – 30), after addition of Coelenterazine 400a, divided by the light emitted by the RLuc8 donor constructs (410 nm – 80). The background signal is subtracted from that BRET ratio using the donor-only negative control where only the RLuc8 plasmid is transfected into the cells.When the BRET is measured on the Envision instrument with our described configuration of filters, the ratio of the raw data at 515 and 410 nm typically gives BRET signals in the range 0–40. However, when the BRET is read on a CLARIOstar, the BRET ratio is around 100 times lower than for the Envision. For the titration curves, the results are analyzed by nonlinear regression assuming a hyperbolic model. BRET ratio calculation for competition assays In order to compare the effect of a competitor (macromolecule or small molecule) on a RAS PPI, we normalize the BRET ratio, calculated as above, to a negative control included in the experiment (usually no competitor or non-RAS binder for macromolecules and DMSO for small molecules). The normalized BRET ratio is the calculated as follows: _BRETcompound/BRETDMSO_ Å~ 100 or _BRETanti-RASmacromolecule/BRETnegative controlmacromolecule_ Å~ 100 or _BRETanti-RASmacromolecule/BRETno competitor_ Å~ 100; where BRETcompound corresponds to the BRET ratio for the compound-treated cells, BRETDMSO corresponds to the BRET ratio for DMSO-treated cells, BRETanti-RAS macromolecule corresponds to the anti-RAS macromolecule (iDAb RAS or DARPins) transfected cells, BRETnegative control macromolecule corresponds to the negative control macromolecule transfected cells (non-RAS binder, e.g., anti-LMO2 iDAb) and BRETno competitor corresponds to the cells not transfected with a competitor. Total GFP2 and RLuc8 signals are used to control the protein expression from each transfected plasmid.

REAGENTS AND SOLUTIONS BRET-based donor and acceptor empty vectors See Figures 4 and 5 and DNA sequences in Supplementary Figure 1 (see Internet Resources) Cell lysis buffer for immunoblot 1% SDS (20% SDS solution is stored at room temperature) 10 mM Tris.Cl, pH 7.4 (1 M Tris.Cl, pH 7.4, solution is stored up to 1 year at 4°C) Protease inhibitors (Sigma, cat. no. P8340-1ML; store aliquots at −20°C) Phosphatase inhibitors (Thermo Fisher, cat. no.1862495, store at 4°C) Store up to one day at 4°C EGF solution preparation (1000Å~), 50 µg/ml Dissolve 100 µg of EGF in 2 ml of sterile phosphate-buffered saline (PBS; to be performed under aseptic condition with a laminar flow hood). Divide into 20 µl aliquots and store up to 1 year at −20°C.

COMMENTARY
Background Information
Several methods allow the study of PPI dynamics in living cells. The Resonance Energy Transfer (RET) is the most commonly used method for that purpose where a transfer of energy occurs from a donor to an acceptor molecule placed in a close proximity. Indeed, some Fluorescence RET (FRET) based- RAS biosensors have already been developed to study RAS activity in living cells (Nakamura, Aoki, & Matsuda, 2005). They have been mostly used to reveal the spatiotemporal dynamic regulation of RAS activity (Nakamura et al., 2005).

However, these biosensors show some limitations because of the excitatory light used as initial source of energy for the excitation of the donor including issues of phototoxicity, photobleaching of the fluorophores, and background auto-fluorescence from the sample (Boute, Jockers, & Issad, 2002; Pfleger & Eidne, 2006). Therefore, we decided to use another RET method, the bioluminescence RET (BRET) that does not rely on an excitatory light as initial energy for the donor. Indeed, the BRET uses a Renilla Luciferase or variants [such as the RLuc8 (De, Loening, & Gambhir, 2007)] as donor. The BRET2 is the first modified version of the original BRET assay (called BRET1). It uses the coelenterazine 400a (or DeepBlueCTM), a chemical derivative of the coelenterazine that shifts the maximal light emission of RLuc to 395 nm (De et al., 2007; Pfleger & Eidne, 2006). The BRET2 method has been improved with the generation of the RLuc variant RLuc8 that increases the BRET ratio by a 5.5-fold (De et al., 2007). Appropriate acceptors for the BRET2 are GFP2 or the GFP10 as their large excitation peaks at 395 nm overlap well with the RLuc8 emission. Their emission maximum is at 510 nm. The major advantage of BRET2 over BRET1 method lies in the better spectral separation of the donor and acceptor emission peaks (_ 115 nm). This implies less bleed through at the acceptor emission maximum and therefore a lower background (Bacart, Corbel, Jockers, Bach, & Couturier, 2008; Couturier & Deprez, 2012; Pfleger & Eidne, 2006). If the RLuc8 donor fusion molecule catalyzed the coelenterazine 400a and the GFP2 acceptor fusion molecule is located less than 10 nm from the donor, a transfer of energy will occur from the donor to the acceptor. This method has been widely used to study PPI (Bery et al., 2018; Felce et al., 2017; Mercier et al., 2002) and also PPI inhibition by small molecules (Beautrait et al., 2017; Corbel et al., 2017; Corbel et al., 2011; Lavoie et al., 2013; Mazars & Fahraeus, 2010; Quevedo et al., 2018) or macromolecules (Bery et al., 2018; Guillard et al., 2017; Spencer-Smith et al., 2017). It offers several advantages: it is a very sensitive technique as it is based on luminescence, there is no background signal from cellular autofluorescence. It is also a reversible and dynamic method and the BRET experiments are easy to perform, to record and to analyze, as there is no need of specialized equipment. The quantification of the response is possible, which is useful for the comparison of different inhibitors (e.g., for structureactivity- relationship studies). Additionally, the BRET signal is a ratio (see Basic Protocol 5). This means that it is insensitive to variables such as cell number and transfection efficiency. Consequently, the BRET allows the detection of weak BRET efficiency. Even though the implementation of the BRET assay is straightforward, the development and optimization of the BRET probes are critical to get a strong and reliable BRET signal.

Critical Parameters and Troubleshooting

The position of the donor and acceptor moieties (i.e., RLuc8/GFP2) on the protein of interest (POI) and the linker length between these moieties and the POI need to be optimized in order to get the highest BRET signal (Table 11). If structural data is available between the two proteins of interest, such as RAS/CRAF, therefore a rational design of the BRET probes can be performed. However, if no structural data is available, a test and try method should be used to determine the best combination. Another critical parameter that requires optimization is the donor and acceptor quantity to express for the visualization of a PPI inhibition. As explained in the introduction of the Basic Protocol 2, it is essential to avoid the titration of an active inhibitor due to an excess of acceptor or the donor/acceptor pair expressed within the cell. When small molecules are tested in the BRET competition assay a negative control BRET pair should be used to eliminate any inhibitors that would interfere with the assay and select genuine inhibitors of PPI. A small molecule could emit in the GFP channel or perturb the RLuc8 that could modify the BRET signal or also modify the cell viability. This is why we use a non-relevant BRET pair (LMO2/iDAb LMO2dm) as negative control for any BRET competition assay. A negative control is also mandatory when

macromolecules are used in a competition assay, the best negative control being a similar protein than the macromolecule tested (iDAb control for iDAb RAS, DARPin control for DARPin RAS, . . .). The BRET is a sensitive method with a low background; however, the residual background should be removed from the BRET signal of a genuine interaction using proper negative control. This background is determined by expressing the donor plasmid only in cells.

Statistical Analyses

The BRET statistical analyses are performed using Prism 7.0c (GraphPad Software). Data are typically presented as mean Å} SD and statistical analyses are performed with a one-way ANOVA followed by Dunnett's post-hoc tests. This test compares the mean of the BRET ratio from each sample to the mean of the BRET ratio of the negative control.

Anticipated Results

The donor saturation assay will show whether two proteins interact specifically together. The results are typically analyzed by nonlinear regression assuming a hyperbolic model (see Basic Protocol 5) and from that model two important parameters can be calculated: the BRET50 and the BRETmax . The BRETmax represents the total number of donor/acceptor complex and the distance between the donor and the acceptor within the dimer. The BRET50 corresponds to the acceptor/ donor ratio necessary to reach 50% of the BRETmax . It is a reflection of the relative affinity of the acceptor fusion for the donor fusion proteins (Lavoie et al., 2013). The BRET competition assay with macromolecules usually gives better PPI inhibition that can reach more than 90% of inhibition, while with small molecules, the inhibition is around 50% to 70% maximum. This can be explained by the size of the macromolecules Bery and Rabbitts (_ 10 to 20 kDa), much bigger than the small molecules (< 1 kDa). Obviously, these percentages of inhibition are also dependent on the level of expression of the macromolecules within the cells, on the uptake of the small molecules by the cells and on the potency of these inhibitors (affinity, binding geometry, . . .).

Time Considerations

The BRET donor saturation assay is generally performed over a period of four days and includes cell seeding, transfection, and expression over a period of 24 hr, BRET plate preparation and plate reading. The BRET2 competition assay is generally performed over a period of three to four days and includes cell seeding, transfection and expression over a period of 24 hr, BRET plate preparation, compounds incubation (over a period of 3 to 20 hr), and plate reading. This time can be extended to another two days if a western blot analysis is performed. Overall, the BRET plate preparation can take anywhere from 30 min to two hours depending on the number of samples and plates being run. The time it takes to read a full 96-well plate can take from 15 to 25 min depending on the plate reader.

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