Bioluminescence Resonance Energy Transfer (BRET)-Based Synthetic Sensor Platform for Drug Discovery

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Bioluminescence resonance energy transfer (BRET) is a technique that analyzes protein-protein interactions (PPIs). The unique feature of BRET delineates that the resonance energy is generated by the resonance energy donor, *Renilla* luciferase by the oxidative decarboxylation of coelenterazine substrate. BRET is superior to FRET where issues such as autofluorescence, photobleaching, and light scattering can occur. Recently, BRET has been applied to design synthetic biosensors for monitoring autophagy in vivo and in vitro. Here, we report the methods for constructing a biosensor of human HsLC3a as a probe for autophagy biogenesis and the optimization of the intramolecular BRET assay that allows for high-throughput screening of chemical modulators of autophagy. User-friendly working interface with the BRET-based synthetic sensor of HsLC3a makes drug discovery easy and amenable for high-throughput. The BRET protocol described here could be easily applicable to generate other biosensors for monitoring PPIs by measurement of intermolecular BRET. © 2017 by John Wiley & Sons, Inc.

Keywords: autophagy • bioluminescence resonance energy transfer (BRET) • drug discovery • HsLC3a • HsATG4 • and luciferase • protein-protein interactions (PPIs)

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INTRODUCTION

Most biological processes require the interaction of proteins in which the function and activity of a protein is determined by other proteins (Fields & Song, 1989; Phizicky & Fields, 1995). Understanding protein-protein interactions (PPIs) facilitates investigation of molecular events in the cell and identification of new therapeutic approaches against diverse diseases. Development of bioluminescence resonance energy transfer (BRET) technology began with the discovery of green fluorescent protein (GFP) and the diversification of the use of bioluminescent proteins (Shimomura, Johnson, & Saiga, 1962). Investigation of intermolecular protein interactions with measurable data in vitro, in vivo, and in real time is possible with the BRET technology, compared to other approaches such as yeast two-hybrid system, bimolecular fluorescence complementation (BiFC), and co-immunoprecipitation (Woo, 2008). In the case of the two-hybrid screening, its main limitation is the high false positive rate (Deane, Salwinski, Xenarios, & Eisenberg, 2002). In BiFC, the reconstitution of the functional fluorescent protein does not dissociate back to its halfmers; therefore failing to render the goal of real time assay (Villalobos,

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Naik, & Piwnica-Worms, 2007). The co-immunoprecipitation has disadvantages where signals often result from protein aggregation artifacts and it cannot be performed in living cells or native conditions (Persani, Calebiro, & Bonomi, 2007). Fluorescence resonance energy transfer (FRET) assay works similarly to BRET in which both approaches rely on Förster resonance energy transfer but limitation of FRET includes autofluorescence, photobleaching, and light scattering to the image background resulting in weakening the measurement of Förster resonance energy transfer from the energy donor to its compatible acceptor (Dragulescu-Andrasi, Chan, De, Massoud, & Gambhir, 2011). When analyzing PPIs in living cells with FRET, spectral mixing is an issue depending on the fluorescent protein used and requires complex methods to correct this issue (Sekar & Periasamy, 2003).

BRET is a highly sensitive approach that offers several advantages over other PPI assays. BRET system consists of *Renilla* luciferase (RLUC) or a variant as a resonance energy donor instead of a fluorescent protein in FRET. Since the energy acceptor, a fluorescent protein, receives the resonance energy from the oxidative decarboxylation of the coelenterazine substrate by the luciferase, this eliminates the false-positive signals and the requirement for an external illumination to excite the energy donor as in FRET (Salahpour et al., 2012; Woo, 2008). The efficiency of the resonance energy transfer depends on several factors: wavelength overlap between the emission maximum of the donor and the absorption spectrum of the acceptor, spatial configuration between the energy donor and its acceptor, and quantum yield of the energy donor (Villalobos et al., 2007). More importantly, the resonance energy transfer is dependent on the distance between the donor and the acceptor where the efficiency of the resonance energy transfer is the inverse correlation with the sixth power of the distance between the BRET tags. Therefore, the donor must be in close proximity in order to excite the acceptor (Xu, Piston, & Johnson, 1999; Woo, 2008). Due to RLUC being used as the resonance energy donor in BRET, a photon of blue bioluminescence is released after returning of the internal energy status of the luciferin from its excitatory state to ground state once the coelenterazine substrate is decarboxylated by the luciferase, thus indicating the importance of RLUC's kinetic properties to generate the resonance energy in the BRET system. In efforts to improve experimental resolution of BRET technology, engineered RLUC variants with better kinetic characteristics have been adopted as an energy donor (Woo, 2008; Woo & von Arnim, 2008). The emission maxima of RLUC is around 470 nm, which overlaps well with the absorption spectra of the energy acceptors such as yellow fluorescence protein (YFP) or Citrine variant of YFP. The change in light spectrum can be monitored with a luminometer and the BRET ratio of the yellow fluorescence to the blue luminescence measured can be calculated (Xu et al., 1999; Woo, 2008). To investigate PPIs, a protein of interest is fused in frame with the donor and the interacting counterpart protein is fused to the acceptor. If the two proteins are interacting with each other within the Förster radius (~50 Å), resonance energy is successfully transferred upon the decarboxylation of coelenterazine by RLUC and yellow fluorescence and blue luminescence signals can be recorded (Xu et al., 1999; Woo, 2008). Since coelenterazine is nontoxic and diffuses readily into animal and plant cells, BRET can be used to measure real time PPIs in live cells (Salahpour et al., 2012; Subramanian et al., 2006).

The BRET technology can be applied to basically any intracellular signaling that involves PPIs including investigation of protease activity (Woo, Park, & Dinesh-Kumar, 2014), kinase activity (Siddiqui, Cong, Daimon, Martin, & Maudsley, 2013), and G protein-coupled receptors (GPCR) (Eidne, Kroeger, & Hanyaloglu, 2002). Recently, BRET applications have been used to study dynamic cellular processes by the development of biosensors or the screening for pharmacologically active compounds (Salahpour et al., 2012). Several BRET versions have been described: BRET1, BRET2, BRET3, and BRETn (Bacart, Corbel, Jockers, Bach, & Couturier, 2008). Each version is based on

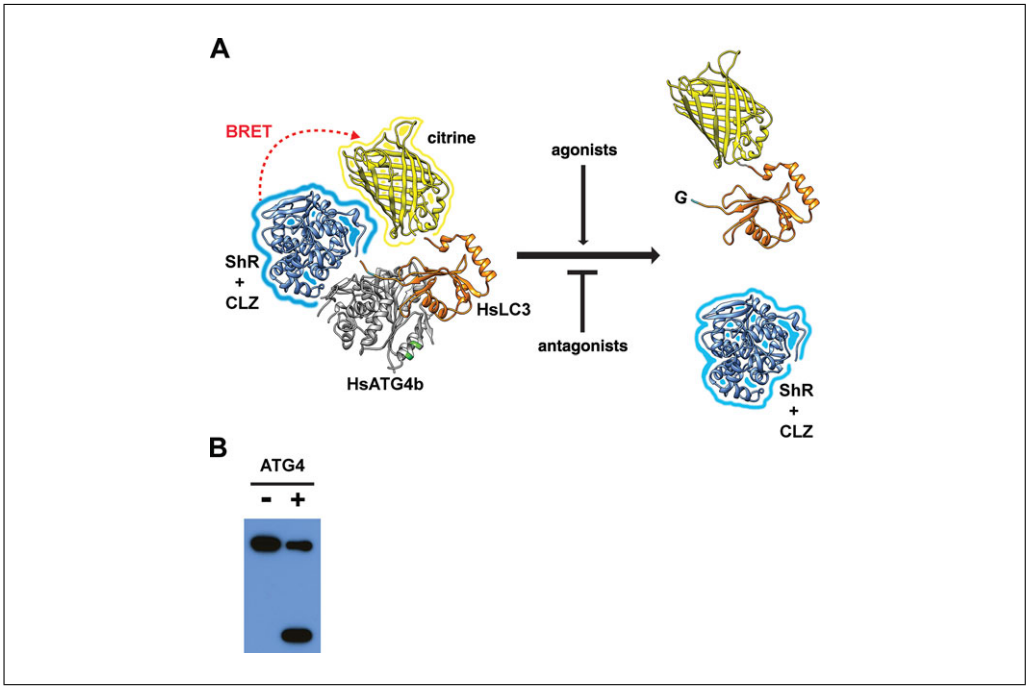

Figure 19.30.1 Schematics of Bioluminescence Resonance Energy Transfer (BRET)-based HaLC3a synthetic sensor for screening chemical modulators of autophagy. (**A**) Citrine as a resonance energy acceptor and ShR as a resonance energy donor are translationally fused to the amino and the carboxyl terminus of human LC3a (HsLC3a), respectively. Cleavage of the HsLC3a BRET-sensor by HsATG4b at the conserved glycine in HsLC3a, results in the separation Citrine-HsLC3a and ShR. The cleavage results in a lower BRET ratio compared to the full-length intact sensor. The BRET-based synthetic sensor can be used for high-throughput screening. Upon incubation of HsATG4b with agonists, the BRET ratio decreases and incubation with antagonists increases the BRET ratio. ShR, (SuperhRLUC), a variant of human codon optimized *Renilla* luciferases; CLZ, coelenterazine, a substrate of ShR. (**B**) In vitro cleavage assay of HsLC3a-sensor. Full-length BRET sensor is detected by immunoblot with anti-ShR antibody in the absence of HsATG4b. The full-length BRET sensor and the cleavage byproduct, ShR, are detected in the presence of HsATG4b. Plus and minus indicate presence and absence of HsATG4b, respectively.

different substrate applications and different compatible energy donors and acceptors. The diverse BRET technology can serve as an adaptable and efficient platform for the accelerated discovery of therapeutic drugs that can be aimed at curing cancers, neurodegenerative diseases, or targeting cellular pathways (Bacart et al., 2008; Salahpour et al., 2012).

Here, we describe the construction of a human ortholog of yeast AuTophaGy 8 (ATG8), LC3a (HsLC3a) BRET sensor, its implication in autophagy biogenesis, and the optimization of the intramolecular BRET assay that allows for high-throughput drug screening. Autophagy is a highly conserved biological process that involves a multi-step formation of autophagosomes that carries intracellular cargoes to the lysosome or vacuole for degradation and/or recycling (He & Klionsky, 2009; Hayward & Dinesh-Kumar, 2011; Jin, Liu, & Klionsky, 2013; Kaur & Debnath, 2015; Wen & Klionsky, 2016). Recent studies have suggested that autophagy functions in both cancer cell survival and tumor suppression (Rubinsztein, Codogno, & Levine, 2012). Therefore, modulating the complex mechanism of autophagy is a new target for cancer therapy (Zhang, Li, Ouyang, Liu, & Cheng, 2016). Membrane phospholipid and cargo selection in autophagy biogenesis are mediated by core components such as ATG4 and ATG8. ATG4 is a cysteine protease that is essential in the biogenesis of autophagosomes by processing the ubiquitin-like ATG8 proteins at the evolutionally conserved glycine residue at the carboxyl terminus of ATG8s (Ohsumi, 2001). Therefore, ATG4-mediated processing of ATG8 is an excellent target to apply

intramolecular BRET for monitoring the kinetics of ATG4 because the result of its enzymatic reaction induces separation of ATG8 (Woo et al., 2014). Due to the importance of HsLC3a in the formation of autophagosomes, we have designed a BRET-based synthetic sensor with HsLC3a to monitor the kinetic activity of HsATG4b. In the BRET-based synthetic sensor of HsLC3a, Citrine fluorescence protein and modified *Renilla* luciferase (ShR) are fused to the amino- and to the carboxyl-terminus of HsLC3a, respectively (Seo et al., 2016; Woo et al., 2014). The full-length of HsLC3a is in the middle between the energy donor and the acceptor (HsLC3a-sensor) (Fig. 19.30.1A). In principle, efficient BRET occurs within the full-length HsLC3a-sensor without HsATG4b cleavage because of the close proximity between the energy donor and the acceptor. However, BRET will be reduced upon HsATG4b-mediated processing of HsLC3a-sensor. Therefore, the molecular status of HsLC3a-sensor can be monitored by intramolecular BRET measurement. In addition, the BRET results can be validated by a different detection method such as *in vitro* cleavage assay (Seo et al., 2016; Woo et al., 2014) (Fig. 19.30.1B). Together, we describe the development of a unique HsLC3a-sensor that is suitable for monitoring autophagy biogenesis by BRET. Furthermore, this BRET-based synthetic sensor can be easily adaptable to investigate autophagy biogenesis in other eukaryotic organisms and to discover small molecules or chemical modulators of autophagy biogenesis in any species of interest.

BASIC PROTOCOL

CHEMICAL LIBRARY SCREENING FOR HsATG4b-MEDIATED PROCESSING OF HsLC3a-SENSOR

This protocol describes high-throughput screening with the BRET-based HsLC3a synthetic sensor to identify small molecules or chemical modulators of autophagy.

Materials

Purified HsATG4b and HsLC3a-sensor proteins (Seo et al., 2016; Woo et al., 2014)
Ice
Coelenterazine (CLZ; see recipe)
Phosphate-buffered saline (PBS) solution, pH 7.4 (e.g., VWR Life Science, cat no. E504-500 ML)
Dimethyl sulfoxide (Sigma-Aldrich, cat no. 472301-500 ML)
Small-molecule chemical library (user defined)

PCR 8-tube strips (e.g., USA Scientific, cat no. 1402-2700)
PCR tube rack
Aluminum foil
Multichannel pipette capable of handling 1 to 100 µl volumes
Multichannel pipette reservoir trough (e.g., VWR, cat no. 89094-662)
96-well white, flat bottom, tissue culture treated, polystyrene plates (e.g., Corning, cat no. 3917)
Adhesive aluminum sealing film for 96-well plates (e.g., ThermoFisher Scientific, cat no. AB0626)
Centrifuge
Plate reader (e.g., TECAN Infinite 200)
Excel software (Microsoft Office, Microsoft)

Preparation of high-throughput chemical library screening

1. Make a dilution of 50 ng/µl of purified HsATG4b and HsLC3a-sensor with PBS. Distribute 30 µl of HsATG4b into each of an 8-linked PCR tubes. Do the same for HsLC3a-sensor in another 8-linked PCR tube. Place the tubes in a PCR tube rack and keep them in ice.
2. Prepare a 100 µM concentration of CLZ by diluting it with ethanol and cover the tube with aluminum foil because CLZ is light sensitive. Store it on ice.

3. Add PBS into a multichannel pipette reservoir trough and transfer 92 µl PBS into each well of a 96-well assay plate using a multichannel pipette
4. Add 2 µl DMSO with a multichannel pipette into columns 1 and 12. These columns will be used as the reference. Chemicals can be added to the remaining wells. Cover the assay plate with a sheet of adhesive aluminum sealing film. Centrifuge the plate for 1 min at 200 × *g*, room temperature.
5. Add 2 µl of 50 ng/µl of HsATG4b from step 1 using a multichannel pipette to each well of 96-well plate containing PBS and DMSO from the left side of the plate to right by using 8 of the 12 tips. Gently mix the solution by moving it back and forth. and incubate it for 10 min at room temperature.

   NOTE: *For chemical screening, chemical compounds can be incubated with HsATG4b.*
6. Add 2 µl of 50 ng/µl of HsLC3a-sensor from step 1 to each well with a multichannel pipette from left to right. Gently mix the solution by tapping the plate with your hands and incubate for 20 min at room temperature. At 15 min, distribute the 100 µM CLZ into an 8-linked PCR tube.
7. Add 2 µl of the 100 µM CLZ (final concentration, 2 µM) to each well from left to right.
8. In the TECAN plate reader, open the script (refer to script parameters section below).
9. Eject the tray and place the plate into the TECAN plate reader.
10. Click RUN to initiate the program. The program will read the BRET measurement by recording amount of blue luminescence and Citrine fluorescence that are emitted.
11. Once the reading is finished, run the program two more times. Export all data into Excel.

    NOTE: *If an input records OVER instead of a value, rerun the program.*
12. "Label 1" represents yellow fluorescence and "Label 2" represents the blue luminescence. Insert a function in Excel to divide the values of "Label 2" by "Label 1" to calculate their ratio. Apply the function to all of the dataset.
13. Repeat step 11 for the two repeat experiments.
14. Average the ratios of the three experiments that correspond to each well.

    The ratios from the wells of columns 1 and 12 serve as reference controls.
15. Determine the highest and lowest ratio of the reference group. They will serve as parameters for selecting hit chemicals.

    Chemical inhibitors have a ratio that is 0.1 higher than the highest ratio in the control and chemical activators have a ratio that is 0.1 lower than the lowest ratio in the control. For example, if the maximum and the minimum among control BRET ratios are 1 and 0.9, chemical hit molecules with the BRET ratios above 1.1 and below 0.8 are collected as inhibitors and activators, respectively.

IN VITRO CLEAVAGE ASSAY BY SDS-PAGE AND IMMUNOBLOTTING

ALTERNATE PROTOCOL

This protocol is performed to confirm the "hit" chemicals that are identified from the BRET measurement in the Basic Protocol.

Materials

Purified HsATG4b and HsLC3a-sensor proteins (Seo et al., 2016; Woo et al., 2014)
Dimethyl sulfoxide (DMSO; e.g., Sigma-Aldrich, cat no. 472301-500 ML)

Small-molecule chemical library (user defined)
2× Laemmli sample buffer (e.g., Bio-rad cat no. 1610737)
SDS-PAGE gel (e.g., Mini-Protean TGX Precast Gels 10%; Bio-rad, cat no. 4561034)
Protein marker (e.g., Spectra Multicolor Broad Range Protein Ladder, cat no. 26634)
10× Tris/Glycine/SDS Buffer (see recipe)
Cathode buffer (see recipe)
Anode I and II buffers (see recipes)
Methanol (e.g., Fisher Chemical, cat no. A412Sk-4)
Distilled water
5% and 3% nonfat milk (see recipes)
Primary antibody anti-*Renilla* luciferase, clone 5B11.2 (e.g., EMD Millipore, cat no. MAB4400)
Phosphate-buffered saline (PBS) solution, pH 7.4 (e.g., VWR Life Science, cat no. E504-500 ML)
Tween-20 (e.g., VWR Life Science, cat no. 0777-1 liter)
Secondary antibody, anti-Mouse IgG (whole molecule)-peroxidase antibody produced in rabbit (e.g., Sigma-Aldrich, cat no. A9044-2 ML)
SuperSignal West Pico Chemiluminescent Substrate (e.g., ThermoFisher Scientific, cat no. 34077)

50-ml conical tubes (e.g., Corning, cat no. 430290)
Centrifuge
Water bath
Floating tube rack
Electrophoresis chamber (e.g., Mini-PREOTEAN Tetra Vertical Electrophoresis Cell; cat no. 1658004)
Electrophoresis power supply (e.g., Bio-Rad, PowerPac HC High-Current Power Supply)
Orbital shaker (e.g., VWR Orbital Shaker, cat no. DS-500E)
Plates (any plastic 4 in × 4 in container that can hold membrane and liquid solutions)
PVDF transfer membranes (e.g., Immobilon, cat no. IPVH00010)
Plate tray
Thick blot filter paper (e.g., Bio-Rad, cat no. 1650921)
Transfer system (e.g., Trans-Blot Turbo Transfer System; Bio-Rad, cat no. 1704155)
Tweezers
Paper towels
Sheet protector non-glare simple loading 8.5 in × 11 in (e.g., Office Depot, cat no. 535038)
Beakers (small and large)
1.5-ml microcentrifuge tubes
X-ray film (e.g., Research Products International, cat no. 248300)
X-ray film cassette
Film developer machine with dark room for film developing
Stir bars
Hot plate

Quantification of the cleavage byproducts to estimate HsATG4b activity

1. Dilute HsATG4b to 6.25 ng/µl and dilute HsLC3a-sensor to 100 ng/µl using PBS.
2. Add 16 µl (~100 ng) of HsATG4b to each 1.5-ml microcentrifuge tube.

3. Add 2 µl DMSO to a test tube. This will be used as the control. 2 µl of small molecules/chemicals can be added to the remaining test tubes. Mix the test tubes by tapping and centrifuge the test tubes for 1 min at 1000 × *g*, 22°C. Incubate the test tubes for 10 min at room temperature.
4. Add 2 µl (200 ng) HsLC3a-sensor into the test tubes starting with the control and then in the order of which the small molecules/chemicals were added. Mix the tubes by tapping and centrifuge for 1 min at 1000 × *g*, 22°C. Incubate for 20 min at room temperature.
5. Prepare a boiling water bath using a hot plate.
6. Add 20 µl of 2× Laemmli sample buffer to each test tube in order. Mix and place the tubes in a floating tube rack. Place the rack in the boiling water bath for 5 min.
7. Take out the floating tube rack, mix the contents of the test tubes by tapping with hands, and centrifuge for 1 min at 1000 × *g*, 22°C.
8. Prepare the electrophoresis chamber with the protein gel. Fill the chamber with 1× Tris/Glycine/SDS buffer.
9. Load 20 µl of protein marker into the first well. Then load 20 µl from each test tube in order starting with the control. Close the chamber with the cover and run the electrophoresis at constant current, 60 mA.

   Prepare 5% and 3% nonfat milk solution.

10. Stop when the dye front reaches to 1 cm from the bottom of the gel and take out the protein gel. Carefully remove the stacking layer and transfer the gel to a plate with cathode buffer. Incubate the gel with cathode buffer on an orbital shaker at 60 rpm for at least 15 min.
11. Prepare a sheet of transfer membrane by cutting a piece that is similar to the size of the protein gel. Place the membrane in a plate tray. Treat the membrane with methanol (by pouring 20 ml methanol to cover the entire surface). Discard the methanol in a separate container. Wash the membrane three times, each time with 20 ml distilled water (enough to submerge the membrane). Add 20 ml Anode II buffer to the membrane and place it on the shaker at 60 rpm for at least 5 min.
12. Prepare transfer by soaking two blotting membranes in anode I buffer, one thick blot filter paper in anode II buffer, and three blotting membranes in cathode buffer.
13. Place the following from the anode bottom to the cathode cover of the transfer system, in this order (Fig. 19.30.2):

a. Two thick blot filter papers with anode I buffer
b. One thick blot filter paper with anode II buffer
c. PVDF transfer membrane
d. Protein gel
e. Three thick blot filter papers with cathode buffer

14. Close the transfer apparatus with the cathode cover. Insert the transfer apparatus to the Trans-Blot Transfer System and run at 25 V and 1 A for 30 min (standard conditions).
15. Pour about 25 ml of 5% nonfat milk solution into a plate tray. Once the transfer is complete, carefully add the membrane to the plate tray with the protein side facing up. Incubate for 1 hr on the shaker at room temperature.
16. Pour about 25 ml of 3% nonfat milk into a plate tray and add 2.5 µl of anti-*Renilla* luciferase antibody and place it on the shaker to mix evenly. Use a pair of tweezers

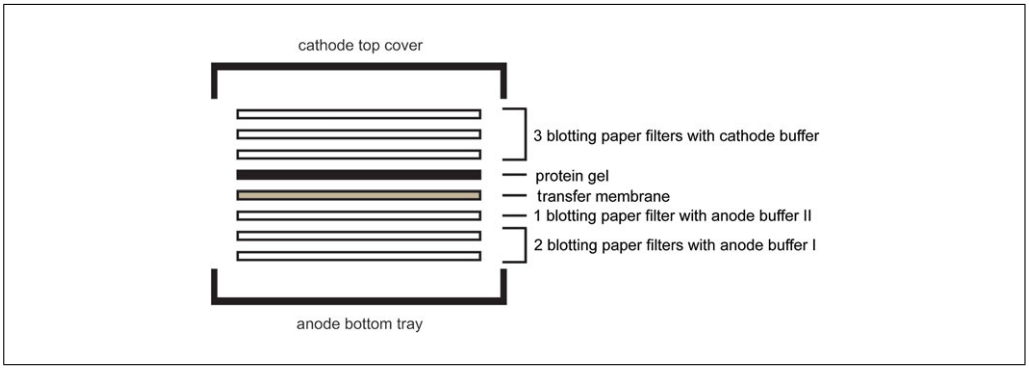

Figure 19.30.2 Schematic of protein transfer set up. A diagram shows protein transfer set up in the transfer apparatus. To assemble a protein transfer sandwich, follow this order of set up from the anode bottom tray to the cathode top cover: two blotting paper filters soaked with anode buffer I, one blotting paper filter with anode II, a PVDF transfer membrane, a protein gel, and three blotting papers soaked with cathode buffer.

and grab the membrane by the edge from the 5% milk plate tray from step 15, remove excess milk from the membrane by dabbing the edge on a paper towel, and place it in the plate tray with 3% milk with the primary antibody. Incubate the membrane on the shaker at 60 rpm for 1 hr at room temperature.

17. Transfer the membrane to a new plate tray with 25 ml PBS with 0.1% (v/v) tween-20 and place it on the shaker for 5 min. Pour the used PBS with 0.1% tween-20 (v/v) into a beaker and add fresh 25 ml of PBS with 0.1% tween-20 (v/v) and wash the membrane for 5 min. Repeat this for 5 more times.
18. Transfer the membrane into a plate tray with 25 ml of 3% milk and 2.5 µl of anti-Mouse IgG-peroxidase antibody. Place it on the shaker at 60 rpm and incubate for 1 hr at room temperature.
19. Repeat step 17 to wash the membrane again.
20. Cut the sheet protector on the edges that are connected. The sheet protector should be able to open like a book. Transfer the membrane with the protein side facing up onto one side of the sheet protector.
21. Mix 500 µl of SuperSignal West Pico Stable Peroxide solution and 500 µl of SuperSignal West Pico Luminol/Enhancer Solution in a 1.5-ml microcentrifuge tube. Mix the contents of the tubes by tapping with hands. Add all contents to the surface of the membrane.
22. Close the sheet protector and make sure there are no air bubbles inside the sheet. Transfer the sheet protector to an X-ray cassette and close it.
23. Develop the image in the dark room with an X-ray film. Place the film on top of the protector sheet with the membrane inside. Expose the film at various times to get the desired results.

SUPPORT PROTOCOL

This protocol describes how to analyze the film by comparing the intensities of the signals with ImageJ (Schindelin et al., 2012).

Materials

Film (see Alternate Protocol)
Image scanner
ImageJ program (e.g., Fiji program, NIH)
Excel program

1. Scan the film using an image scanner and save the image. Open the ImageJ program and open the image file. Under the Edit option, select Invert.
2. Click and drag to make a rectangular shape that encloses the largest white blot on the image. Drag the enclosed rectangle to a black area of the image and press "m" on the keyboard. This data will serve as the background. Then drag the rectangle to the control's blot and press "m". Repeat this with subsequent blots. The data will be stored in a new window. Copy and paste the numbers into Excel.
3. In a new column, subtract the Mean of each data to the background data.
4. In a new column, divide each difference with the difference of the control. The control should have a ratio of 1. Subsequent ratios are interpreted as signals that are X times stronger or weaker than the control. This is used to confirm the inhibition or activating nature of chemicals.

REAGENTS AND SOLUTIONS

Use deionized, distilled water, unless otherwise indicated.

Anode I buffer

20 ml of 1.5 M Tris·Cl, pH 10.4
10 ml methanol
70 ml distilled water
Store up to 2 months at room temperature

Anode II buffer

1.7 ml of 1.5 M Tris·Cl, pH 10.4
10 ml methanol
88.3 ml distilled water
Store up to 2 months at room temperature

Cathode buffer

1.92 M glycine
1.7 ml of 1.5 M Tris·Cl, pH 9.4
10 ml methanol
81.7 ml distilled water
Store up to 2 months at room temperature.

Coelenterazine (CLZ)

Make 100 µM dilution of CLZ (Santa Cruz Biotechnology, cat no. 55779-48-1) with ethanol
Protect CLZ from light by wrapping with aluminum foil and store up to 1 month at −20°C

Nonfat milk, 3% and 5%

5% and 3% Milk:
Add 5 g of milk powder (nonfat dry milk omniblok; Americanbio, cat no. AB10109-01000) to 100 ml of 1× phosphate-buffered saline (PBS) with 0.1% (v/v) tween-20 and stir to mix at room temperature. Prepare 3% milk similarly using 3 g of milk powder.

Tris/Glycine/SDS buffer, 1×

900 ml distilled water + 100 ml of 10× Tris/Glycine/SDS Buffer (e.g., Bio-rad, cat no. 1610732)

COMMENTARY

Background Information

While the BRET technique is very useful in studying PPIs, FRET can be used as an alternative approach. As discussed above, the FRET technique has some limitations. BRET is theoretically better than FRET when quantifying resonance energy transfer (Xu et al., 1999). However, BRET sensors need to be optimized before starting high-throughput screening experiments to make sure that the replicate control experiments produce similar data. This encompasses the optimization of reaction using BRET sensors and the estimation of Z' factor that represents quality of high-throughput screening. Since BRET-sensors are highly sensitive, human error including pipetting errors is a concern that contributes to the variability of BRET ratios between replicates. Furthermore, because the assay is time sensitive due to the continuous cleavage activity, efforts to reduce time gap between samples is advisable. Each reaction is supposed to receive 20 min of incubation to allow cleavage of the sensor. Without consistency in pipetting timing, the reactions in each well can contribute to various incubation periods, which can lead to variable data. Moreover, luminescence can be dim which requires a sensitive light-measuring device. An alternative method suitable for drug screening similar to this BRET-based system is the thermal shift assay. Thermal shift assay is the use of dyes to monitor thermal denaturation of proteins with sensitive fluorescence detection and to determine protein stability using real-time PCR instruments (Huynh & Partch, 2015). This application can be used to discover new protein-ligand interactions similar to the BRET method described.

Critical Parameters

The concentrations of the proteins must be 50 ng/µl when using the BRET method. The final concentration of CLZ is 2 µM. For the Alternate Protocol, the concentration of HsATG4b must be 6.25 ng/µl and HsLC3a-sensor be 100 ng/µl.

TECAN program parameters:

- Mode: Dual Color Luminescence
- Plate: Corning 96 Flat Bottom White Polystyrol
- Shaking (Orbital) Duration: 3 sec
- Shaking (Orbital) Amplitude: 1 mm
- Filter 1: BLUE1
- Integration Time 1: 100 msec
- Filter 2: GREEN1
- Integration Time 2: 100 msec
- Settle Time: 0 msec

Adjusting the filters can change the range of wavelengths that the machine will read. When the filter set is chosen for BRET measurement, the emission maxima of 470-nm and 530-nm wavelength should be considered for blue luminescence and yellow fluorescence, respectively. Changing the integration times to a longer period can result in a higher background noise. The duration of the shaking can be increased but it may have little effect on the readings because the time that it takes to read the data does not affect the ratios dramatically. Because an "OVER" indicates out of range of a luminescence value detected by the luminometer, the short reading time is better to record the luminescence value. The ratios usually stay consistent over time.

Troubleshooting

Poor signals in X-ray films

If the immunoblots show little and no signal even with longer exposure, add 5% SuperSignal West Femto Chemiluminescent Substrate to the 500 µl of SuperSignal West Pico Chemiluminescent Substrate (25 µl of Femto Stable Peroxide and 25 µl Femto Luminol/Enhancer + 475 µl Pico Stable Peroxide and 475 Pico Luminol/Enhancer). Add more Femto, if necessary. However, background signals become more visible when Femto is used. Furthermore, protein concentration can affect the signal. The optimal amount of HsATG4b to HsLC3a-sensor is 1:2. As more HsATG4b is added, more HsLC3a-sensor is cleaved and the signal of the full-length HsLC3a-sensor will become weaker while the cleaved signal will be stronger.

Anticipated Results

There should be two rows of bands with different molecular weights. The bands at the top row represent the uncleaved HsLC3a-sensor and its size should be around 80 kDa (Fig. 19.30.1B). The bottom bands represent the cleaved HsLC3a-sensor and it should be around 35 kDa (Fig. 19.30.1B). The lane of DMSO should be included as the reference in all cleavage assays. An effective inhibitor should prevent cleavage of HsLC3a-sensor so that the signal with low molecular weight is less than the reference. On the other hand, an activator of a chemical/drug molecule increases cleavage. Therefore, the signal should be stronger than the reference. If the signal is

similar to the reference, then the chemical is ineffective in modulating HsATG4b-mediated cleavage of HsLC3a-sensor.

Time Considerations

The membrane can be stored for extended periods of time during various points of the protocol: during the blocking stage with the 5% milk, during primary antibody incubation, and during secondary antibody incubation. The membrane should be stored on a shaker at 60 rpm at 4°C. The entire basic protocol may take 1 hr per plate. The alternate protocol may take up to 6 hr.

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Conflicts of Interest

The authors declare there is no conflict of interest.

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