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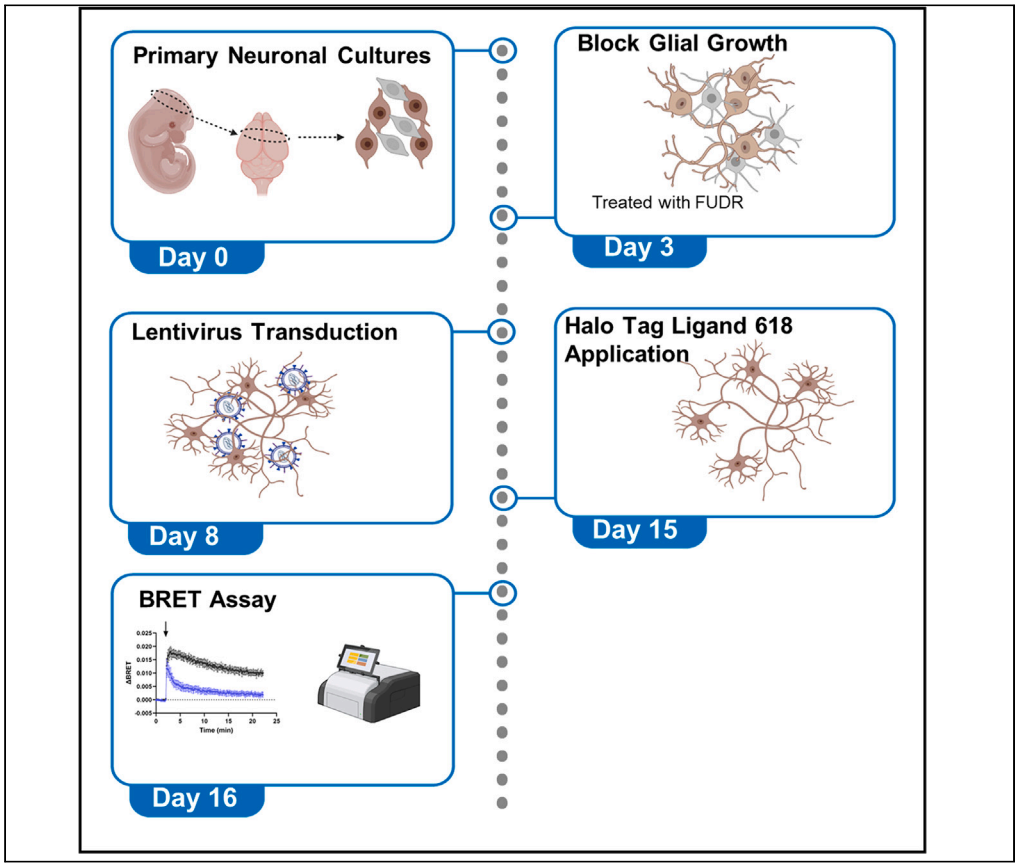
Protocol

Application of bioluminescence resonance energy transfer assays in primary mouse neuronal cultures



Bioluminescence resonance energy transfer (BRET) is widely employed for real-time monitoring of G protein-coupled receptor activity, interactions, and trafficking in heterologous cell lines, yet its use in neuronal systems remains limited. Here, we present a protocol to apply BRET assays to primary neuronal cultures from mouse embryos. We describe steps and key concepts for generating plasmid constructs and lentivirus preparations, plating and lentiviral transduction of primary cultured neurons in 96-well plates, and BRET data collection and analysis.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Instructions for plating and lentiviral transduction of neurons in 96-well plates

BRET data collection from primary neuronal cultures

Quick BRET data analysis using a supplied template

Data comparing agonist-induced OXTR interactions in HEK293 cells and neurons

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Application of bioluminescence resonance energy transfer assays in primary mouse neuronal cultures

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SUMMARY

Bioluminescence resonance energy transfer (BRET) is widely employed for real-time monitoring of G protein-coupled receptor activity, interactions, and trafficking in heterologous cell lines, yet its use in neuronal systems remains limited. Here, we present a protocol to apply BRET assays to primary neuronal cultures from mouse embryos. We describe steps and key concepts for generating plasmid constructs and lentivirus preparations, plating and lentiviral transduction of primary cultured neurons in 96-well plates, and BRET data collection and analysis. For complete details on the use and execution of this protocol, please refer to George et al.[1]

BEFORE YOU BEGIN

Background

Bioluminescence Resonance Energy Transfer (BRET) is based on the transfer of energy from a bioluminescent donor to a fluorescent acceptor when the two are in close proximity. This technique has found extensive use in real-time tracking of protein-protein interactions,[2,3] protein trafficking through cellular compartments,[4] and monitoring cell signaling[5,6] in live cells. In particular, this method is being widely used to characterize G protein coupled receptor (GPCR) biology. There are many advantages of BRET over standard immunocytochemical methods including real-time tracking of receptors in live cells with much better temporal resolution. The BRET assays track the molecular proximity at dimensions of 10–20 nm that is beyond the scope of colocalization-based imaging assays. Immunocytochemical methods also require large amounts of the epitope-tagged receptor to be expressed (in cases where there is absence of good extracellular motif-targeting antibodies) that can produce overexpression artifacts. This problem may be reduced in BRET because the use of highly efficient energy donor allows the expression of the receptor in only small amounts.[7] Despite these advantages, the application of this method has largely been restricted to heterologous cell lines with not much use described in neuronal systems. With increasing recognition that GPCR signaling and trafficking show cell-specific features, it will be useful to harness the power of BRET for investigating neuronal GPCRs within their native cellular milieu. We have recently developed and adapted BRET assays for application in primary neuronal cultures that allowed investigations of oxytocin receptor in neurons.[1] Here, we provide a detailed protocol for application of BRET assays in primary neuronal cultures to characterize GPCR signaling, interactions and trafficking (Figure 1). These assays should be easy to adapt to investigate neuronal proteins other than GPCRs including ionotropic receptors, synaptic scaffold proteins and synaptic cell adhesion molecules.

The protocol described here is based on the use of NanoBRET, which is a recently developed version of BRET that uses NanoLuc luciferase as the energy donor.[7,8] The advantage of NanoLuc is its high

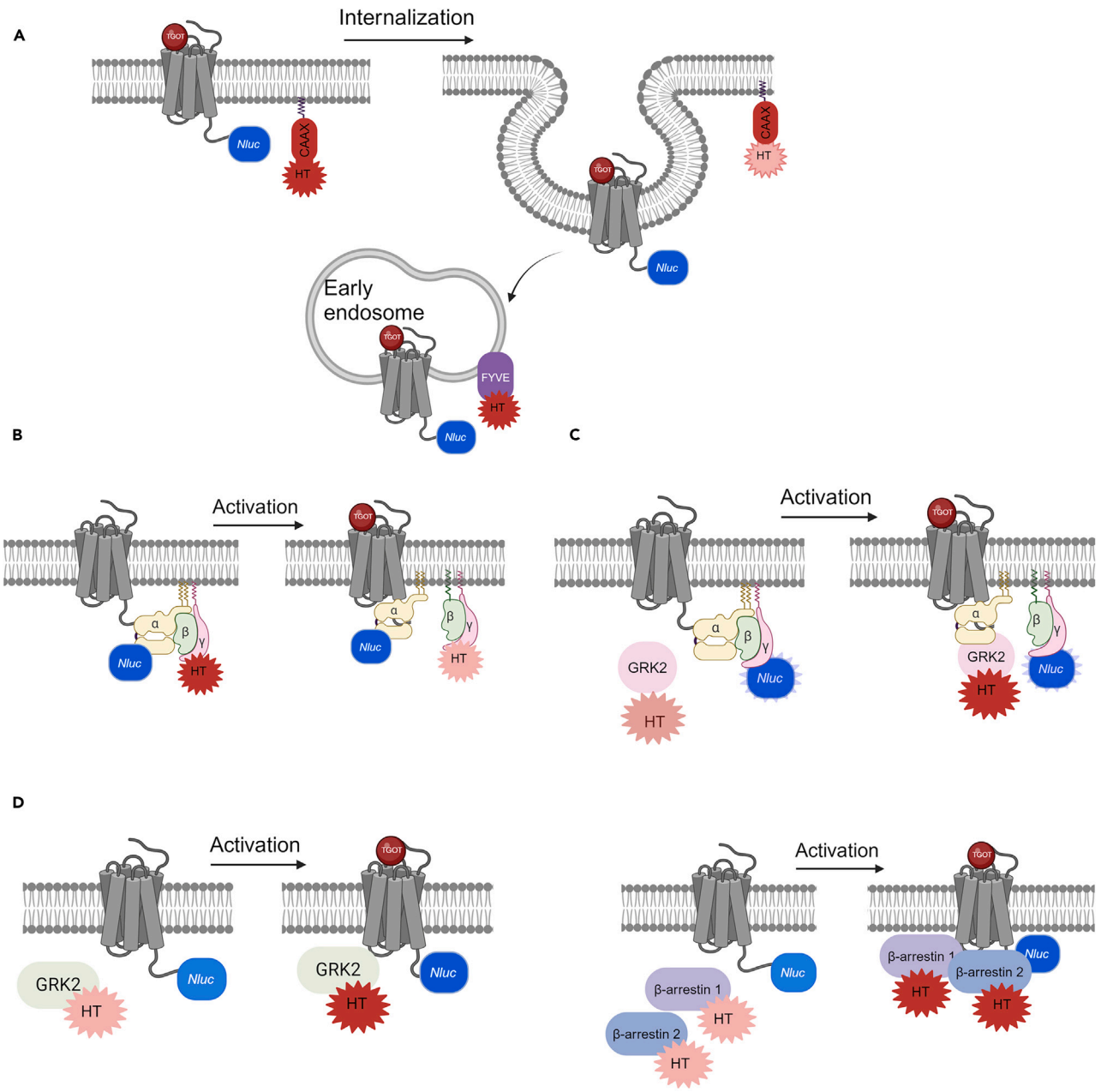

Figure 1. Schematics showing the design of the BRET assays applied successfully in primary neuronal cultures

(A) Receptor trafficking assays. NanoLuc (Nluc) fused to GPCR at its C-terminus generates BRET with HaloTag (HT)-CAAX at the plasma membrane. Following agonist binding, the receptor is internalized, leading to a decrease in the BRET ratio. The recruitment of internalized GPCR to early endosomes is tracked with a BRET assay in which nanoLuc is fused to the C-terminus of the receptor, whereas HaloTag is attached to 2xFYVE sequence box that directs it to early endosomes.

(B) G protein activity assay based on separation of Gα from Gβγ. Following agonist binding to the GPCR, Gα-NanoLuc separates from Gβγ-HaloTag.

(C) G protein activity assay based on binding of Gβγ with GRK2/3. Following agonist binding to the GPCR, freed Gβγ-NanoLuc come in close proximity to GRK2/3.

(D) Recruitment assays. NanoLuc is fused to the C-terminus of the GPCR and HaloTag is attached to GRK2 (left), and β-arrestin-1 or β-arrestin-2 (right). Upon agonist binding to the receptor, GRK2/β-arrestin-1/β-arrestin-2-HaloTag is recruited to GPCR-NanoLuc leading to increase in the BRET ratio. The figure is adapted from George et al.[1] with modifications.

bioluminescence that allows it to be used at low expression levels, and small size (compared to older luciferases) that reduces interference with the biological function of the fused protein. The commonly used energy acceptor in NanoBRET assays is HaloTag combined with HaloTag 618 Ligand. The combination of NanoLuc (emission maximum at 460 nm) and HaloTag/618 Ligand (emission maximum at 618 nm) provides the advantage of a wide spectral separation between energy donor and acceptor, thus improving sensitivity. A common variation is the use of YFP or Venus as the energy acceptor that allows commonly available YFP/Venus tagged proteins to be used in NanoBRET assays instead of generating new HaloTag-fused products.[9]

Institutional permissions

All animal experimentation must be approved by the institutional animal use and care committee and be conducted in accordance with institutional animal use and care guidelines. When working with live cells, it is essential to conduct all procedures under sterile conditions in a certified biosafety cabinet. Work involving lentivirus should be approved by an institutional biosafety committee and performed in accordance with the Biosafety Level 2 (BSL2) guidelines.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Lentivirus: L301HaloTag-CAAX	George et al.[1]	N/A
Lentivirus: L301HaloTag-2x FYVE	George et al.[1]	N/A
Lentivirus: L301Syn HA-OXTR-NanoLuc	George et al.[1]	N/A
Lentivirus: L301 β-arrestin-1-HaloTag	George et al.[1]	N/A
Lentivirus: L301β-arrestin-2-HaloTag	George et al.[1]	N/A
Lentivirus: L301GRK2-HaloTag	George et al.[1]	N/A
Chemicals, peptides, and recombinant proteins		
5-Fluoro-2′-deoxyuridine	Sigma	Cat# F0503
B-27 Plus supplement (50×)	Thermo Fisher Scientific	Cat# A3582801
Boric acid	Sigma	Cat# 6768
Cell culture grade water	Corning	Cat# 25-055-CV
Deoxyribonuclease I from bovine pancreas	Sigma	Cat# D5025
DMEM with 4.5 g/L glucose and sodium pyruvate without L-glutamine	Corning	Cat# 15-013-CV
FuGENE 6 transfection reagent	Promega	Cat# E2691
GlutaMAX supplement	Thermo Fisher Scientific	Cat# 35050061
HBSS (10×), no calcium, no magnesium, no phenol red	Thermo Fisher Scientific	Cat# 14185052
HEPES (1 M)	Thermo Fisher Scientific	Cat# 15630080
Hexadimethrine bromide (polybrene)	Sigma	Cat# H9268
Isoflurane solution	Covetrus	Cat# 11695067772
Neurobasal Plus medium	Thermo Fisher Scientific	Cat# A3582901
Penicillin-Streptomycin (10,000 U/mL)	Thermo Fisher Scientific	Cat# 15140122
Poly-L-lysine	Sigma	Cat# P2636
Sodium tetraborate dehydrate	Sigma	Cat# B9876
(Thr^4,Gly^7)-Oxytocin (TGOT)	Bachem	Cat# 4013837
Trypsin (2.5%), no phenol red	Thermo Fisher Scientific	Cat# 15090046
Uridine	Sigma	Cat# U3003
Critical commercial assays		
NanoBRET HaloTag 618 ligand	Promega	Cat# G9801
NanoBRET Nano-Glo substrate	Promega	Cat# N1571
NanoBRET Nano-Glo detection systems	Promega	Cat# N1661
QuickTiter Lentivirus Titer Kit	Cell Biolabs	Cat# VPK-107
Experimental models: Cell lines		
HEK293	ATCC	CRL-1573

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Mouse: C57BL6/J	The Jackson Laboratory	Stock #000664, RRID:IMSR_JAX:0 00664
Recombinant DNA		
L301HaloTag-CAAX	George et al.[1]	CAAX sequence from Addgene #79574
L301HaloTag-2x FYVE	George et al.[1]	2xFYVE sequence from Addgene #140047
L301Syn HA-OXTR-NanoLuc	George et al.[1]	mouse OXTR GenBank: NM_001081147
L301HA-OXTR-NanoLuc	This paper	mouse OXTR GenBank: NM_001081147
L301 β-arrestin-1-HaloTag	George et al.[1]	mouse β-arrestin-1 GenBank: NM_177231
L301β-arrestin-2-HaloTag	George et al.[1]	mouse β-arrestin-2 GenBank: NM_001271358
L301GRK2-HaloTag	George et al.[1]	mouse GRK2 GenBank: NM_001290818
Software and algorithms		
GraphPad Prism	GraphPad	https://www.graphpad.com/scientific-software/prism/
Fiji (ImageJ)	NIH	https://imagej.nih.gov/ij/
Microsoft Office	Microsoft Office	https://www.office.com/
Other		
Agilent BioTek Synergy Neo2 hybrid multi-mode microplate reader	Agilent	Neo2
GFP and luminescence. Dual PMT filter cube	Agilent	Cat# 1035101
NanoBRET. Dual PMT filter cube	Agilent	Cat# 1035074
96-well Assay plate	CoStar	Cat# 3917
Steriflip PVDF 0.45 μm filter unit	Sigma	Cat# SE1M003M00

MATERIALS AND EQUIPMENT

Equipment

The specialized equipment needed for this protocol is as follows. Alternative equipment with comparable features may be used in their place.

Agilent BioTek Synergy Neo2 plate reader

Emission filters: 450/610 nm (NanoLuc/HaloTag 618 Ligand).

Measurement height: 6 mm.

Measurement time: 0.2 s.

Gain: 135.

Costar assay plate, 96 well

Color: White.

⚠ CRITICAL: Use white opaque plates to reduce loss of signal through leak or adsorption to plate walls and to prevent interference by signal from surrounding wells.

Material: Polystyrene.

Tissue culture treated: Yes.

Cell culture media and stock solutions

- Neuronal culture plating medium: Neurobasal plus medium supplemented with 2% B27 plus, 2 mM GlutaMAX, 100 U/mL penicillin/streptomycin, and 5% heat-inactivated horse serum. Store at 2°–8°C for a maximum of 6 weeks.

- Neuronal culture maintenance medium: Neurobasal plus medium supplemented with 2% B27 plus, 2 mM GlutaMAX, and 100 U/mL penicillin/streptomycin. This medium contains no serum. Store at 2°–8°C for a maximum of 6 weeks.
- HEK293 cell maintenance medium: High-glucose DMEM with sodium pyruvate supplemented with 2 mM GlutaMAX and 10% heat-inactivated horse serum. The addition of antibiotics should be avoided as they reduce lentiviral titers. Store at 2°–8°C for a maximum of 8 weeks.
- 5-Fluoro-2′-Deoxyuridine stock solution: 100× stock solution contains 500 μM 5-Fluoro-2′-Deoxyuridine and 500 μM uridine in DMEM. Store at −20°C for a maximum of one year.
- Dissection solution: Hank's Balanced Salt Solution supplemented with 10 mM HEPES, pH 7.4. Prepare on the day of use.
- Digestion solution: Dissection solution with 0.25% Trypsin. Prepare on the day of use.
- Trypsin Neutralization solution: Dissection solution with 40 U/mL DNase. Prepare on the day of use.
- Poly-L-lysine solution: 1 mg/mL poly-L-lysine in 0.1 M Borate buffer (pH 8.5). Filter-sterilize and store at 2°–8°C for a maximum of 6 months. This can be used for coating plates multiple times, saving on the cost.

STEP-BY-STEP METHOD DETAILS

Generation of plasmid constructs

Timing: 1 week

1. Utilize standard cloning methods to fuse relevant protein-expressing cDNA with NanoLuc and HaloTag coding sequences to generate BRET plasmid constructs.

Note: Careful consideration should be given to the location in the protein where NanoLuc or HaloTag are fused so as not to interfere with the trafficking, localization and/or biological function of the protein. In general, tags are inserted at the C-terminus of β-arrestins and GPCR kinases (GRKs) and at the N-terminus of Rab proteins.[1] It is recommended to insert a glycine/serine linker between the protein of interest and tag.

Generation of lentivirus preparations

Timing: 10 days

Lentiviral vectors provide an efficient method of delivery for BRET constructs in primary neurons, transducing almost 100% of cells. It is recommended to use the 3rd generation lentiviral system that has a better safety profile than the earlier 2nd generation vectors. Among the lentiviral shuttle vectors suitable for use in primary neuronal cultures are those utilizing the human ubiquitin C promoter or the human synapsin promoter. The human ubiquitin C promoter drives ubiquitous expression of the protein of interest, while the human synapsin promoter ensures selective expression in neurons. Several such vectors are available in the Addgene repository.

2. Culture of HEK293T cells.
   a. Thaw a fresh vial of HEK293T cells and transfer the contents to a 15 mL tube containing warm HEK293 maintenance medium. Centrifuge at 200 *g* for 5 min. Remove the medium.
   b. Resuspend the cell pellet in 1 mL warm medium and transfer to a 10 cm dish with 9 mL medium.
   c. Maintain cell confluence between ∼30% and ∼80%.
   d. Split cells by trypsinization 1:6-1:8 every other day; for weekend maintenance, use a 1:8-1:10 split ratio.
   e. Thaw a fresh vial of HEK cells every 2 months and limit passages to 15–20 before thawing a new vial.

3. Transfection of HEK293T cells for lentivirus production.
   a. Day 1 - Plating.
      i. Split 80% confluent cells 1:3–4 into T75 flasks, aiming for 60%–70% confluence by the next morning.
      ii. Consider coating flasks with poly-L-lysine for improved cell adherence.
   b. Day 2 - Transfection.
      i. Warm DNA, medium, and Fugene6 to room temperature.
      ii. Prepare DNA mix by mixing 10 μg of lentiviral shuttle vector and 5 μg of each packaging vector.
      iii. Make Fugene6 mix by adding 75 μL of Fugene6 to 3 mL of DMEM (no FBS). This constitutes DNA:Fugene6 ratio of 1:3. Incubate for 5 min at room temperature.
      iv. Add the DNA mix to the Fugene6 mix a little bit at a time. Incubate at room temperature for 35–45 min.
      v. After incubation, pipette mixture onto cells in the T-75 flask and swirl flask to distribute the mixture. Place the flasks in the 37°C, 5% CO_2 incubator.
   c. Day 4 - Collection and concentration.
      i. Wet a sterile, 0.45 μm PVDF membrane filter unit with HEK293 maintenance medium.
      ii. Collect virus-containing media from the flask gently and transfer to a 50 mL tube.
      iii. Centrifuge at 1000 *g* for 5 min at 4°C.
      iv. Collect the supernatant and pass through the wet PVDF filter unit by connecting the filter nozzle to vacuum. Filtering removes cellular debris from the supernatant.
      v. Ultracentrifuge filtered supernatant at 45,000 g for 90 min at 4°C.
      vi. Decant the supernatant.
      vii. Add DMEM (without any FBS) supplemented with polybrene to centrifuge tubes and store at 4°C.
   d. Day 5 – Harvesting and measuring titer.
      i. Resuspend the concentrated virus and make aliquots of 5–10 μL each in low binding 0.5 mL tubes. Store at −80°C.
      ii. Measure the titer of the virus preparation using a p24 ELISA-based method, according to manufacturer's instructions (https://www.cellbiolabs.com/sites/default/files/VPK-107-lentiviral-titer-p24-elisa-kit.pdf).

Note: This method relies on the detection of p24 protein on the capsid of the lentivirus. We find that the titer obtained from this method is generally a reliable correlate of the degree of infectivity of the virus preparation.

Preparation and maintenance of primary neuronal cultures

Timing: 7 days

We recommend preparing primary neuronal cultures from mouse embryos (E17-E18), belonging to the strain of choice. The cultures prepared from embryonic brain are healthier and survive better compared to postnatal brain-derived neurons. Several detailed protocols to prepare primary neuronal cultures from rodent brains have been published.[10–12] Below we provide an adaptation of published methods that is established in our laboratory for BRET assays.

4. Prepare and maintain primary neuronal cultures from mouse embryos in coated 96-well plates.
   a. Prepare 96-well plates for cell attachment by coating overnight with 1 mg/mL poly-L-lysine in 0.1 M Borate buffer at room temperature.

Note: We limit the preparation to a maximum of 24 wells per 96-well plate. This enables us to collect BRET data with a sampling interval of 10 s. Placing an excessive number of sample wells in a plate reduces the sampling rate at which the data can be collected.

b. On the day of cell plating, wash wells with sterile water three times over a period of 3 h and store at room temperature with wells filled with sterile water. Don't let the wells dry.
c. Euthanize pregnant female mouse with isoflurane inhalation followed by decapitation. Make an incision in the abdominal wall and extract the embryonic pups.
d. Harvest the brains in dissection solution and remove all meninges.
e. Dissect cortical (neocortical and hippocampal) tissue from brains and incubate with a digestion solution containing trypsin for 15 min at 37°C.
f. Incubate the tissue in trypsin neutralization solution containing DNase for 10 min at room temperature.
g. Triturate the tissue in plating medium.
h. Remove water from the poly-L-lysine-coated wells of the 96-well plate by suction just before plating the cells.
i. Plate cells in the wells of 96-well plates at a density of 100,000–120,000 cells per mL of the plating medium. We add cells in the bulk medium and then transfer 200 μL of medium per well which amounts to 20–24,000 cells per well.

Note: Use a P1000 pipette to pipette cells as the large bore of the 1 mL tip reduces damage to the cells.

j. Place the plate in a 37°C, 5% CO_2 incubator. This is day-in-vitro 0 (DIV 0).
k. Replace half of the medium with maintenance medium on DIV 3. Add FUDR at this time point to block glial growth. This approach ensures that sufficient glial cells remain to support the neurons, without overwhelming the cultures.
l. Replace half of the medium in each well with fresh maintenance medium on DIV 7.

Lentivirus transduction and HaloTag 618 ligand application

Timing: 8 days

5. Apply lentivirus containing BRET constructs to primary neuronal cultures on DIV8.
   a. Mix the lentivirus solutions in optimal titers (determined as in the note below) with the maintenance medium and replace half of the existing medium in each well with the virus-containing mixture. It is advisable to apply the virus in duplicate or triplicate for every experimental condition. Ensure to double the number of wells to accommodate HaloTag 618 ligand negative wells (see below in step 5d).

   Note: The amount of the lentivirus that is applied to the primary neuronal cultures depends on the carried construct. For constructs expressing NanoLuc, we typically apply an empirically determined quantity that produces NanoLuc signal intensity (at 450 nm) within the range of 0.5–3 million units (at the specified plate reader settings). This is generally achieved with lentivirus concentrations of 1–2 × 10^5 transduction units/mL. Lower lentivirus titers and thus weaker NanoLuc expression leads to greater variability in serial BRET measurements, while higher expression risks saturating the detector. The optimal amount of lentivirus expressing HaloTag constructs needs to be empirically determined by testing different amounts in relation to a fixed quantity of NanoLuc virus. The optimal amount is the one that produces maximal agonist-induced BRET response. We find that having 5–10 times more HaloTag-expressing virus than NanoLuc expressing virus is suitable for most BRET assays in neuronal cultures.

   b. Place the plate back in the 37°C, 5% CO_2 incubator.
   c. Replace half of the medium in each well with fresh maintenance medium on DIV 12.
   d. One day before the experiment (on DIV 15), remove 150 μL of medium from each well, and add 50 μL of fresh maintenance medium supplemented with HaloTag 618 Ligand to a final concentration of 100 nM. For each experimental condition, ensure that each well receiving the HaloTag 618 Ligand is matched with a corresponding well that does not contain this ligand.

Note: Reducing the amount of medium per well from 200 μL to 100 μL at this stage allows the conservation of HaloTag 618 Ligand and creates space in wells for later application of reagents.

BRET data collection

Timing: 1.5 h

6. BRET data are collected on a plate reader that can simultaneously acquire signal from two channels and is equipped with suitable filters.
   a. On the day of the BRET experiment (DIV 16), prepare the injectors of the plate reader by loading them with PBS supplemented with the appropriate concentration of the agonist, or with PBS alone. The injection solution is prepared to be at 6× the agonist concentration. Prime the injectors to fill them to the tip.
   b. Setup the injection and data acquisition protocol.
   c. If pharmacological reagents need to be applied to activate or inhibit signaling pathways beforehand, add the agent or DMSO to cells and incubate at 37°C, 5% CO_2 for desired time. We apply GRK and G protein inhibitors for 30 min.
   d. Prepare a 5× solution of NanoBRET Nano-Glo substrate in maintenance medium by a 100-fold dilution of the stock.
   e. Add substrate (25 μL of 5× substrate solution) to each well containing 100 μL of medium and incubate for 5 min at room temperature.
   f. Insert the plate in the plate reader.
   g. Measure the donor emission at 450 nm and acceptor emission at 610 nm sampling every 10 s. Collect baseline signal for 4–5 min.
   h. Administer agonist through the injector (25 μL of 6× agonist solution) or PBS.
   i. Collect the post-administration signals for the desired amount of time every 10 s.

BRET data analysis

Timing: 15 min

7. We utilize a Microsoft Excel template for data analysis (Table S1), where one tab contains all data values exported from the plate reader along with BRET ratio calculations, while another tab displays time course graphs generated from the data in the first tab.
   a. Calculate BRET ratio as follows. BRET ratio = (Emission610 / Emission 450) $_{\text{HaloTag ligand}}$ - (Emission610 / Emission 450) $_{\text{no HaloTag ligand}}$.
   b. To determine ΔBRET, first obtain the average of the baseline and then subtract this average from all values.
   c. To calculate normalized BRET, divide all values by averaged baseline.
   d. The choice between using ΔBRET and normalized BRET depends on the BRET assay (see below).
   e. If the administration of PBS induces a significant change in the BRET ratio, the corresponding values must be subtracted from the BRET values obtained from agonist-administered wells before calculating ΔBRET and normalized BRET.
   f. Create a template file and save it.
   g. When analyzing a new experiment, update the existing data in the first tab. This action will automatically update the BRET ratio calculations in the first tab and the graphs in the second tab.

EXPECTED OUTCOMES

We have successfully applied several BRET assays for investigating OXTR biology in primary neuronal cultures.[1] The schematic for selected assays is presented in Figure 1. OXTR can be replaced with other GPCRs to adapt to the needs of individual projects.

Receptor surface localization and internalization

Energy donor: OXTR-NanoLuc. NanoLuc is fused to the intracellular C-terminus of OXTR.

Energy acceptor: HaloTag-CAAX, Lyn11-HaloTag. CAAX box derived from KRAS is fused to the C-terminus of HaloTag to target it to the cell membrane.[13] Alternatively, the first 11 amino acid residues of Lyn (Lyn11) can be fused to the N-terminus of HaloTag for plasma membrane targeting.[14] Lyn11 exhibits partial preference for lipid rafts, whereas CAAX is thought to be excluded from them.

Expected outcomes: The baseline BRET ratio indicates the targeting of OXTR to the cell membrane. Mutations disrupting cell membrane localization of a GPCR decrease the baseline BRET ratio, making it a useful indicator for comparing the relative cell membrane targeting of wild-type and mutant receptor.[4] Agonist application triggers a rapid decrease in the BRET ratio, signifying movement of the receptor away from the CAAX (or Lyn11) labeled plasma membrane, thus indicating internalization. The example data for BRET, ΔBRET, and normalized BRET between OXTR-NanoLuc and HaloTag-CAAX are shown in Figure 2A and tabulated in Table S1.

Receptor recruitment to intracellular membrane compartments

Energy donor: OXTR-nNanoLuc. nNanoLuc is fused to the intracellular C-terminus of OXTR.

Energy acceptor: HaloTag-2xFYVE, HaloTag-Rab5, HaloTag-Rab11. The FYVE domain derived from EEA1 binds to PI(3)P, a phospholipid enriched on the surface of early endosomes. A tandem repeat (2×) of this domain fused to the C-terminus of HaloTag targets it efficiently to early endosomes.[4,15] HaloTag attached to the N-terminus of Rab proteins targets it to the particular Rab compartments.[4]

Expected outcomes: The baseline BRET ratios indicate the constitutive targeting of OXTR to the specific intracellular compartments and are usually quite low. Administration of the agonist increases the ratio with a delay that indicates the time it takes for the activated receptor to reach the particular membrane compartment from the plasma membrane. The example data for BRET, ΔBRET, and normalized BRET between OXTR-nNanoLuc and HaloTag-2xFYVE are shown in Figure 2B and tabulated in Table S1.

Receptor activity

Various BRET assays have been established to measure G protein activation following agonist binding to the GPCR.

Separation of Gα from Gβγ

Energy donor: Gα-nNanoLuc. NanoLuc is inserted at specific internal sites in Gα.

Energy acceptor: HaloTag-Gγ/Venus-Gγ. HaloTag or Venus is fused to the N-terminus of Gγ and co-expressed with Gβ subunit.

Expected outcomes: The binding of the ligand to the GPCR molecule results in separation of GTP-bound Gα from Gβγ. This appears as a decrease in BRET in this configuration.[5]

Interaction of GRK2/3 with released Gβγ

Energy donor: NanoLuc-Gγ. NanoLuc is inserted at the N-terminus of Gγ and co-expressed with Gβ.

Energy acceptor: GRK2/3-HaloTag. HaloTag is fused to the C-terminus of full-length or N-terminal truncated GRK2 or GRK3.

Expected outcomes: The binding of the ligand to the GPCR molecule results in separation of GTP-bound Gα from Gβγ. The released Gβγ binds to GRK2/3 that results in an increase in BRET ratio.[16]

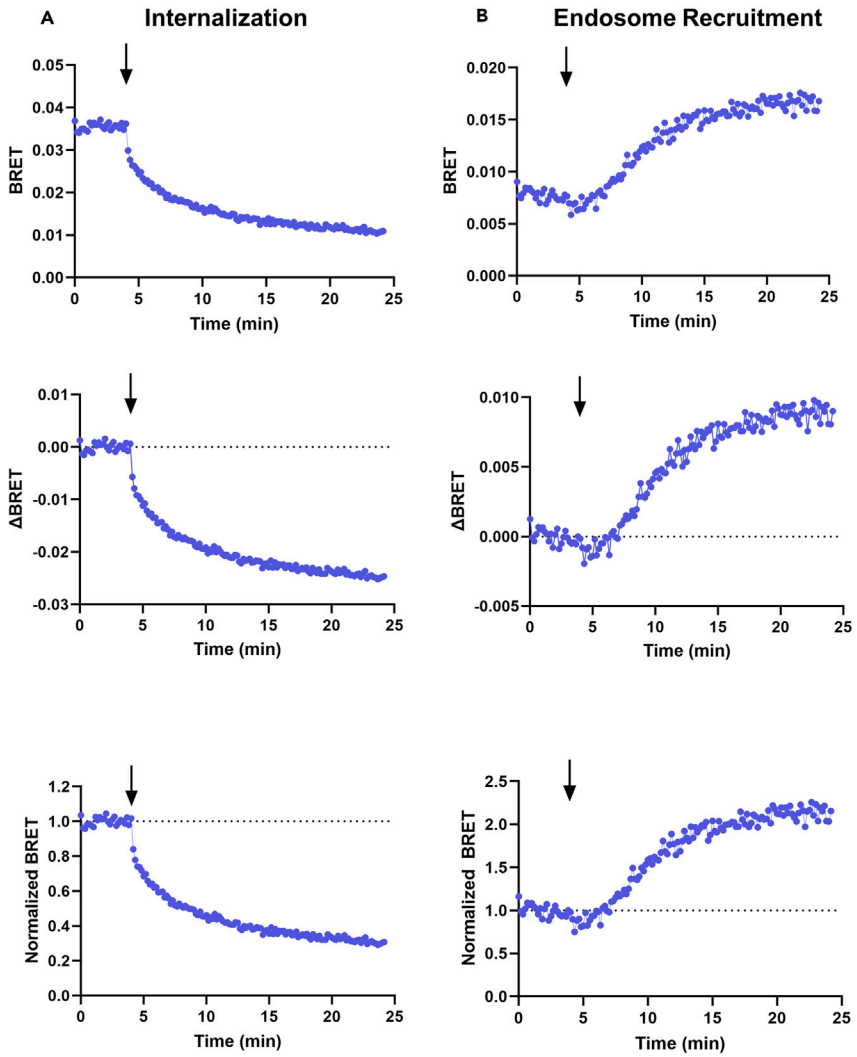

Figure 2. Example time course plots of BRET, ΔBRET and normalized BRET for agonist-induced OXTR internalization and endosome recruitment in neurons

(A) BRET ratio (top), ΔBRET (middle) and normalized BRET (bottom) with OXTR-NanoLuc and HaloTag-CAAX to track agonist-induced receptor internalization. The basal values of BRET in the top graph indicate the level of OXTR surface expression. The arrow indicates application of 200 nM TGOT agonist.

(B) BRET ratio (top), ΔBRET (middle) and normalized BRET (bottom) with OXTR-NanoLuc and HaloTag-2xFYVE to track receptor recruitment to early endosomes. The arrow indicates application of 200 nM TGOT agonist. The data containing replicates were published for similar experiments in George et al.[1].

Interaction of GPCR with G proteins

Energy donor: GPCR-NanoLuc. NanoLuc is fused to the intracellular C-terminus of GPCR.

Energy acceptor: Gγ-HaloTag/Venus. HaloTag or Venus is attached to the N-terminus of Gγ.

Expected outcomes: The binding of the ligand to the GPCR results in the recruitment of G proteins including Gγ to the GPCR in cases where the heterotrimeric G proteins are not preassembled with the receptor. This recruitment manifests as a rise in the BRET ratio.[17]

Biosensing of endogenous GTP-bound Gα

Energy donor and energy acceptor: Unimolecular. Lyn11-NanoLuc (energy donor) is fused to YFP (energy acceptor) that is coupled to a detector module.[9] The detector module is specific for each class of Gα.

Expected outcomes: GTP-Gα produced from ligand-GPCR interaction binds with the detector module, inducing a conformational change in the unimolecular sensor, thereby bringing the energy donor and acceptor into closer proximity and increasing the BRET signal.[9]

Intracellular protein (β-arrestins, GRKs) recruitment to GPCR

Energy donor: OXTR-NanoLuc. NanoLuc is fused to the intracellular C-terminus of OXTR.

Energy acceptor: β-arrestin-HaloTag, GRK-HaloTag. HaloTag is attached to the C-terminus of β-arrestin-1 or -2 or GRKs. Similar tagging can be done on other cytosolic proteins whose interaction with the GPCR needs to be investigated.

Expected outcomes: The baseline BRET ratio of OXTR with β-arrestin-1 and -2 is very low indicating little basal interaction. The application of OXTR agonists induces robust increase in BRET. The baseline BRET between OXTR and GRK5/6 is higher compared to GRK2/3 because GRK5/6 is constitutively targeted to the cell membrane through lipid modifications while GRK2/3 remains cytosolic. GRK2/3 are rapidly recruited to neuronal OXTR following ligand binding, while GRK6 (but not GRK5) shows further interaction resulting in increases in BRET ratio. Importantly, we found that the kinetics of recruitment of β-arrestin-1, β-arrestin-2, and GRK2 to OXTR are different in neurons compared to HEK293 cells (Figure 3). These results demonstrate cell-specific differences in OXTR functioning, emphasize caution in interpreting data from heterologous cell lines, and stress the need for studying the GPCRs in native cells.

LIMITATIONS

The primary drawback of BRET assays lies in their inability to provide spatial information regarding the specific cellular location where the change in proximity between tagged proteins occurs. This limitation is particularly pertinent in neurons due to their polarity and the presence of specialized compartments like dendrites, axons and synapses. This limitation can be overcome by incorporating the use of BRET imaging.[18] Commonly, the BRET constructs are introduced in cells by overexpression which can potentially cause mistargeting of the tagged proteins. The use of highly efficient energy donor, NanoLuc, allows the receptor to be expressed in small amounts,[7] thus reducing but not excluding the possibility of overexpression artifacts. This pitfall may be avoided in the future by the development of efficient CRISPR/Cas9-mediated knock-in methods that could be used to insert NanoLuc and HaloTag at the endogenous loci. The current methods do not have the required efficiency to knock-in both tags in sufficient number of cultured neurons for successful BRET. The development of neurons from induced pluripotent stem cells which have been manipulated and selected for knock-in provides a promising way forward to address this limitation.

TROUBLESHOOTING

Problem 1

NanoLuc signal is weak.

Potential solution

Check the health of neuronal cultures plated in parallel on coverslips in transparent 24-well plates by visual inspection, viability stain and/or immunocytochemical labeling of synaptic, dendritic and axonal markers. Further, immunoblot analysis of pooled wells from the 96-well plate can be done. If the health of cultures is good, the density of plated cells or the amount of lentivirus applied to wells may need to be adjusted to improve NanoLuc signal.

Problem 2

Little effect of agonist application on BRET.

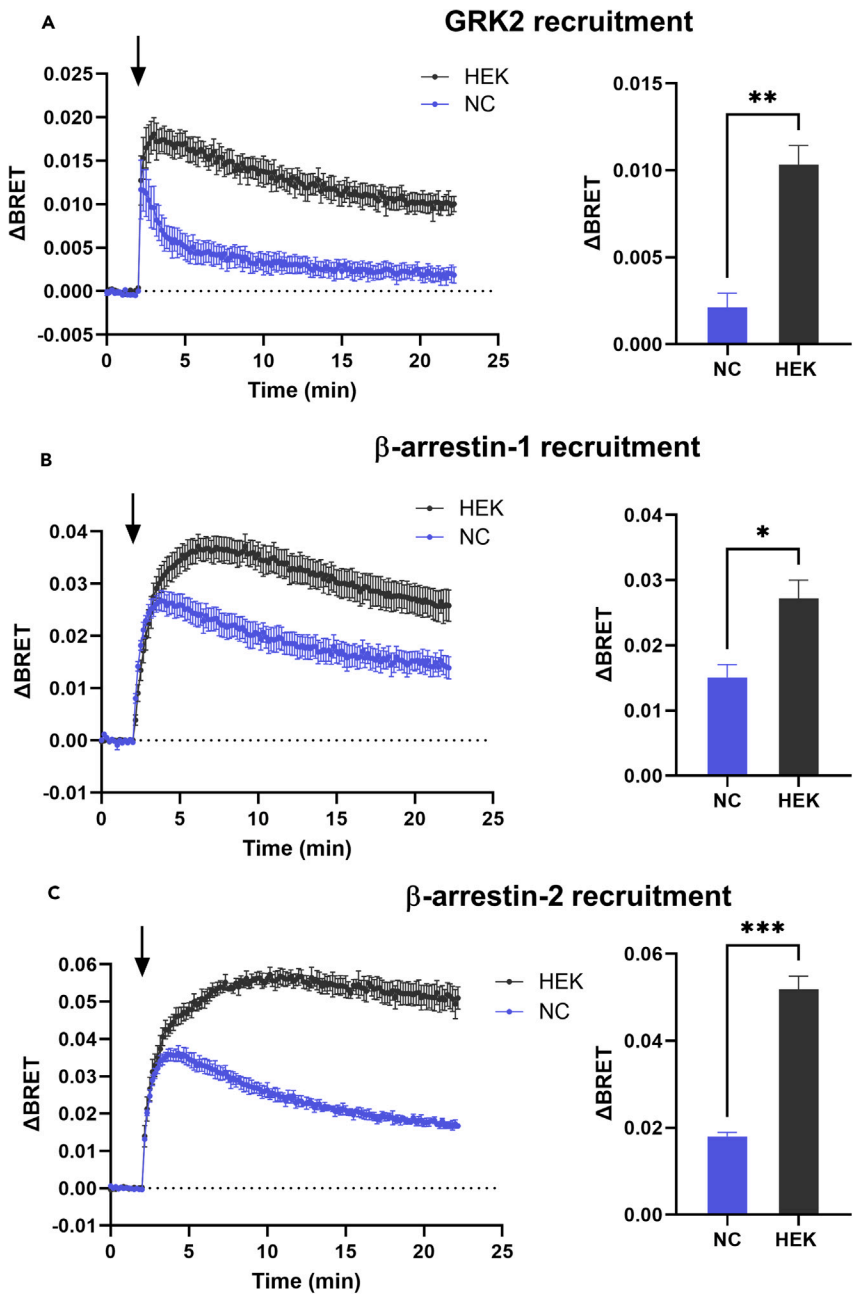

Figure 3. The kinetics of agonist-induced recruitment of GRK2, β-arrestin-1, and β-arrestin-2 to OXTR differ between HEK293 cells and neurons

(A-C) Time course plot of ΔBRET between OXTR-NanoLuc and GRK2-HaloTag (A), β-arrestin-1-HaloTag (B), or β-arrestin-2-HaloTag (C) in HEK293 cells (HEK) and primary neuronal cultures (NC). n = 3–5 per group. The arrow indicates application of 200 nM TGOT. The bar graph shows averaged ΔBRET values at 15–20 min post-receptor activation, demonstrating lesser proximity of GRK2/β-arrestin-1/β-arrestin-2 to OXTR at this time point, which indicates faster dissociation of these molecules in neurons compared to HEK293 cells. Graphs represent Mean ± SEM. Student's t-test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Potential solution

Optimize the ratio of NanoLuc and HaloTag expressing lentivirus by varying the amount of HaloTag expressing virus against a fixed volume of NanoLuc expressing virus. Also, a range of agonist concentrations should be tried based on known affinity values of ligand-receptor binding from literature.

Problem 3

Unequal expression or surface targeting of GPCR mutants.

Potential solution

The expression levels of wild-type and mutant receptors can be analyzed on immunoblots and from NanoLuc signal. Attempts can be made to equalize expression of mutants by altering the amount of expressing lentivirus. The surface expression of GPCR variants can be deduced from the baseline BRET ratio between GPCR-NanoLuc and HaloTag-CAAX. In the case of altered surface expression of mutant GPCR, we recommend the calculation and use of normalized BRET (and not ΔBRET) for BRET assays of internalization.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact: Mohiuddin Ahmad (Mohiuddin-Ahmad@ouhsc.edu).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact: Mohiuddin Ahmad (Mohiuddin-Ahmad@ouhsc.edu).

Materials availability

Requests for resources and reagents will be fulfilled by the lead contact with a completed Materials Transfer Agreement.

Data and code availability

This study did not generate large datasets or code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103228.

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AUTHOR CONTRIBUTIONS

H.T.M.H. and K.G. performed the experiments, optimized assays, and analyzed the data. M.A. conceived the BRET assays, supervised the experiments, and acquired funding. K.G. and H.T.M.H. prepared the figures. M.A. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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