GPCR Activation Assay Protocol (Aequorin-GFP & Gα16)

Objective:
Quantify GPCR activation by measuring calcium-induced luminescence in HEK293 or CHO-K1 cells co-expressing GPCR, Gα16 (promiscuous G-protein coupling to calcium signaling), and an Aequorin-GFP sensor.

Materials & Reagents

Cells:

- HEK293 or CHO-K1 cells stably/transiently expressing:
  - Target GPCR receptor
  - Gα16 protein (universal coupling to Ca^{2+} signaling)
  - Aequorin-GFP calcium-sensitive fusion protein

Reagents:

- Cell culture medium (e.g., DMEM/F12 + 10% FBS + antibiotics)
- Coelenterazine-h (stock solution: 1 mM in ethanol, store at -20°C, protect from light)
- HBSS-based assay buffer:
  - Hank's Balanced Salt Solution with Ca^{2+} and Mg^{2+}
  - 20 mM HEPES, pH 7.4
  - 0.1% fatty-acid-free BSA
- Test ligands (agonists/antagonists, dissolved in assay buffer or suitable solvent)
- Positive agonist controls (known activators of your GPCR)
- Vehicle control (buffer alone or solvent)

Equipment:

- Luminescence-compatible plate reader (e.g., PerkinElmer EnVision, Berthold Mithras, FlexStation)
- White, opaque-bottom 96- or 384-well plates

Step-by-step Protocol

1. Cell Preparation (Day before assay)

- Culture HEK293 or CHO-K1 cells expressing GPCR, Gα16, and Aequorin-GFP until ~80% confluent.
- Harvest and seed cells in white opaque-bottom plates:
    - **96-well plates:** ~40,000 cells/well
    - **384-well plates:** ~15,000 cells/well
- Incubate overnight at 37°C, 5% CO_2 to allow cell adherence and expression stabilization.

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2. Coelenterazine-h Loading (Day of assay)

- **Preparation:**
    - Dilute Coelenterazine-h (1 mM stock) to **5 µM** final concentration in assay buffer (HBSS + 20 mM HEPES + 0.1% BSA, pH 7.4).
    - Protect from light during preparation and use.
- **Loading:**
    - Remove growth medium gently; replace with freshly prepared Coelenterazine-h solution:
        - **96-well:** 100 µL/well
        - **384-well:** 30 µL/well
    - Incubate plates protected from light:
        - Optimal: **1–2 hours at room temperature**

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3. Ligand Stimulation & Luminescent Detection

- **Prepare ligands:**
    - Dilute test compounds, positive controls, and vehicle to desired concentrations in assay buffer.
    - Recommended ligand solutions at 2–3× final assay concentration.
- **Measurement procedure:**
    1. Place plates in luminometer; record baseline luminescence (5–10 sec).
    2. Automatically inject ligand (recommended volumes):
        - **96-well plates:** 25–50 µL injection/well
        - **384-well plates:** 10 µL injection/well
    3. Immediately measure luminescence continuously for **30–60 sec** to capture peak response.

Instrument Settings Recommendation:

- Injection speed: Medium-fast
- Detection interval: ~0.5 sec
- Integration time per reading: 0.5–1 sec

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4. Data Analysis

- Calculate peak luminescence (RLU, relative luminescence units).
- Normalize data to vehicle (fold-induction):

Fold-induction=RLU (ligand stimulation)RLU (vehicle)\text{Fold-induction} = \frac{\text{RLU (ligand stimulation)}}{\text{RLU (vehicle)}}Fold-induction=RLU (vehicle)RLU (ligand stimulation)

- Plot dose-response curves and determine EC_{50} or IC_{50} using sigmoidal curve fitting (GraphPad Prism or similar).

Important Considerations & Tips

- **Gα16 coupling:**
  Gα16 broadly couples diverse GPCR classes to calcium signaling pathways, making it ideal for screening unknown GPCR ligands or orphan receptors.
- **Coelenterazine handling:**
  Always handle under dim or dark conditions; avoid prolonged exposure to ambient light.
- **Cell Density:**
  Optimize for robust signal-to-noise ratio without overcrowding cells.
- **Controls:**
  Always include a known agonist control and vehicle-only controls in every experiment.
- **Transient vs. Stable Expression:**
  Stable cell lines provide higher reproducibility and consistency.