Assay Protocol for Endogenous GPCR Activation in Human Pulmonary Artery Smooth Muscle Cells (PASMCs) Expressing AEQ-GFP in Mitochondria (cAP-0101AEQ-GFP-Mito)

Purpose

This protocol describes a **functional bioluminescence assay** to measure endogenous GPCR activation in **human pulmonary artery smooth muscle cells (PASMCs) stably expressing mitochondrial-targeted AEQ-GFP (cAP-0101AEQ-GFP-Mito)** using **aequorin-based calcium mobilization**.

Materials Required

1. **Cells:** Human PASMCs expressing AEQ-GFP in mitochondria (cAP-0101AEQ-GFP-Mito)
2. **Culture Medium:** SMCs Growth Medium (cAP-24)
3. **GPCR Ligands:** Based on commonly expressed GPCRs in PASMCs (see list below)
4. **Coelenterazine h (or native coelenterazine):** For reconstitution of aequorin (5 µM final concentration)
5. **Assay Buffer:** HBSS + 0.1% BSA (pH 7.4)
6. **$CaCl_2$ (for calcium reintroduction if needed)**
7. **Luminometer (e.g., Berthold MicroLumat LB 96P or equivalent)**
8. **White 96-well or 384-well microplates (luminescence-compatible)**
9. **Trypsin/EDTA (for cell detachment)**
10. **Probenecid (optional, 5 mM, to prevent dye leakage)**

Procedure

Day 1: Cell Seeding

1. **Thaw and Culture Cells:**
    - Recover cells from liquid nitrogen and culture in **SMCs Growth Medium (cAP-24)** at **37°C, 5% CO_2** until ~80% confluent.
    - Passage cells using **trypsin/EDTA** as needed.
2. **Seed Cells for Assay:**

- Detach cells and seed in a **white 96-well plate** at **50,000–80,000 cells/well** in **100 µL growth medium**.
- Allow cells to adhere overnight (~16–24 hr).

Day 2: Aequorin Reconstitution & GPCR Stimulation

1. **Prepare Assay Buffer:**
   - **HBSS + 0.1% BSA** (pH 7.4).
   - (Optional) Add **5 mM probenecid** to prevent dye leakage.
2. **Reconstitute Aequorin with Coelenterazine h:**
   - Dilute **coelenterazine h** in assay buffer to **5 µM** (final concentration).
   - Remove growth medium and add **100 µL/well of coelenterazine h solution**.
   - Incubate **2–4 hr at 37°C** (or **overnight at RT** for better signal stability).
3. **Prepare GPCR Ligands:**
   - Dilute ligands in assay buffer at **10X final desired concentration** (accounting for 1:10 dilution upon addition to cells).
   - **Example concentrations for PASMC-expressed GPCRs:**
     - **Endothelin-1 (ETAR/ETBR):** 10–100 nM
     - **Angiotensin II (AT1R):** 100 nM–1 µM
     - **Thrombin (PAR1/PAR4):** 0.1–1 U/mL
     - **Serotonin (5-HT2B receptor):** 1–10 µM
     - **ATP (P2Y receptors):** 1–10 µM
     - **Prostaglandin F2α (FP receptor):** 100 nM–1 µM
     - **Sphingosine-1-phosphate (S1PR1–3):** 100 nM–1 µM
     - **Urotensin-II (UT receptor):** 10–100 nM
4. **Run the Assay (Luminometer Setup):**
   - Pre-equilibrate plate at **37°C** for 10 min.
   - Set up luminometer to inject **10 µL of 10X ligand** per well (total volume = 110 µL).
   - Measure luminescence **immediately after ligand addition** (1–2 sec intervals for 30–60 sec).

Data Analysis

- **Peak Luminescence:** Measure maximum signal after ligand addition.
- **Area Under the Curve (AUC):** Integrate signal over time for total response.

- **Normalization:** If needed, normalize to baseline or positive control (e.g., ionomycin/Ca^{2+} ionophore for maximum response).

Commonly Expressed GPCRs in Human PASMCs

PASMCs express a variety of endogenous GPCRs, including:

- **Endothelin receptors (ETAR, ETBR)** – Vasoconstriction (ET-1)
- **Angiotensin II receptor (AT1R)** – Vasoconstriction (Ang II)
- **Protease-activated receptors (PAR1, PAR4)** – Proliferation (thrombin)
- **Serotonin receptors (5-HT2B, 5-HT1B)** – Vasoconstriction (5-HT)
- **Purinergic receptors (P2Y1, P2Y2, P2Y6, P2Y12)** – ATP/ADP-mediated signaling
- **Prostanoid receptors (FP, TP, EP1–4)** – PGF2α/TXA2/PGE2 signaling
- **Sphingosine-1-phosphate receptors (S1PR1–3)** – S1P-mediated migration/proliferation
- **Urotensin-II receptor (UT)** – Potent vasoconstrictor (U-II)
- **Muscarinic receptors (M3, M5)** – Acetylcholine-mediated contraction

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Troubleshooting

- **Low Signal:** Increase coelenterazine incubation time or concentration.
- **High Baseline Noise:** Reduce coelenterazine exposure to light (light-sensitive).
- **No Response:** Verify GPCR expression in PASMCs; test positive control (e.g., ATP or ionomycin).

Conclusion

This protocol allows functional assessment of **endogenous GPCR activation** in **PASMCs (cAP-0101AEQ-GFP-Mito)** via **mitochondrial Ca^{2+}-dependent aequorin luminescence**.