Assay Protocol for Endogenous GPCR Activation in Human Liver Sinusoidal Microvascular Endothelial Cells (LSECs) Expressing AEQ-GFP in Mitochondria (cAP-0012AEQ-GFP-Mito)

Purpose

This protocol describes a functional assay to measure endogenous GPCR activation in **Human Liver Sinusoidal Microvascular Endothelial Cells (LSECs) stably expressing mitochondrial-targeted AEQ-GFP (cAP-0012AEQ-GFP-Mito)** using **aequorin-based bioluminescence calcium mobilization**.

Materials Required

1. **Cells:** Human LSECs expressing AEQ-GFP in mitochondria (cAP-0012AEQ-GFP-Mito)
2. **Culture Medium:** Endothelial Growth Medium (cAP-02)
3. **GPCR Ligands:** Based on commonly expressed GPCRs in LSECs (e.g., endothelin-1, angiotensin II, prostaglandins, serotonin (5-HT), histamine, purinergic agonists (ATP/ADP), VEGF, sphingosine-1-phosphate (S1P))
4. **Coelenterazine h (or native coelenterazine):** For reconstitution of aequorin
5. **HBSS (Hanks' Balanced Salt Solution) + 0.1% BSA (assay buffer)**
6. **$CaCl_2$ (for calcium reintroduction if performing in low-calcium conditions)**
7. **Luminometer (e.g., Berthold MicroLumat LB 96P or equivalent plate reader)**
8. **White 96-well or 384-well microplates (cell culture-treated, luminescence compatible)**
9. **Trypsin/EDTA (for cell detachment)**
10. **Probenecid (optional, to prevent dye leakage if needed)**

Procedure

Day 1: Cell Seeding

1. **Thaw and Culture Cells:**
    - Recover cells from liquid nitrogen and culture in **Endothelial Growth Medium (cAP-02)** 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:
     - **Endothelin-1 (ET-1):** 10–100 nM
     - **Angiotensin II (Ang II):** 100 nM–1 µM
     - **ATP:** 1–10 µM
     - **Histamine:** 1–10 µM
     - **Serotonin (5-HT):** 1–10 µM
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).

Expected GPCR Targets in LSECs

Common endogenous GPCRs in LSECs that may elicit Ca^{2+} responses:

- **Endothelin receptors (ETAR/ETBR)**
- **Angiotensin II receptor (AT1R)**
- **Purinergic receptors (P2Y1, P2Y2)**
- **Serotonin receptors (5-HT2B, 5-HT1B)**
- **Histamine receptors (H1R)**
- **S1P receptors (S1PR1–3)**
- **Prostaglandin receptors (EP1–4, FP, IP)**

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 LSECs; test positive control (e.g., ATP or ionomycin).