Calcium fluorimetry with the FLIPR Calcium 6 kit on FlexStation 3 V.2

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We use this protocol and it's working

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ABSTRACT

This protocol details an experimental procedure used to generate results described in the manuscript Li, A., Liu, S., Huang, R., Ahn, S., & Lefkowitz, R. J. (2023). Loss of biased signaling at a G protein-coupled receptor in overexpressed systems.

1 Plate U2OS-TetOn-AT1R in microplates (Black with clear bottom, lysine- coated Corning 3842) at 15000 cells/well 2 days prior to assay

2 Add doxycycline and optionally PTX to wells 14 hours before replacement with loading buffer

3 Remove one vial of Calcium 6 Assay Reagent (Component A, good for 2 plates) from the freezer and equilibrate to room temperature.

4 Dissolve contents of one Component A vial by adding 10 ml of 1X HBSS Buffer plus 20 mM HEPES. Mix by vortexing (~1-2 min) until contents of vial are dissolved. It is important that contents are completely dissolved to ensure reproducibility between experiments.

5 Remove cell plates from the incubator, Calcium 6 Kit - add an equal volume of loading buffer to each well in the dark (i.e. 120ul HBSS+HEPES + 40ul of Dye/well (4X) for a 96-well plate in the original protocol). Use 11.8ml of HBSS+HEPES +4.8ml of Dye (4X) for one plate. Save the dissolved dye solution in -20.

6 Return plates to the incubator and incubate two hours at 37 C, 5% CO2

7 Optionally, add YM-254890 1 hour prior to reading

8 30 minutes before experiment (1.5 hours into incubation), prepare the reagents (5X, 40ul/well+20ul volume). The ligand needs to be plated in a 96 well with round bottom (The FlexStation will add ligand, shake, and measure sequentially).

9 Turn on FlexStation 30 minutes before experiment and warm up to 37.

10 Place tips and ligand plate

11 After the two-hour incubation period, transfer the assay plate directly to the FlexStation instrument assay plate carriage 10 minutes prior to reading to re-equilibrate temperature, and then run the assay.

12 In an individual well or column of wells, the calcium flux peak(s) should be complete within 1 to 3 minutes after addition. For an entire plate however, the protocol will not complete until all chosen columns are finished. 40-45 min for entire 96 well plate.