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Whole-cell radioligand binding for receptor internalization

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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.

Day 1

1 Grow cells on 150 mm dish to ~70% confluency

2 Wash twice with 10 mL PBS

3 Detach cells with 1.5 mL trypsin-EDTA + 10.5 mL media. Trypsinize for 5 minutes and check under microscope for complete detachment; tap dish if necessary

4 Count cells with hematocytometer and dilute collected cells to 164,000 cells/mL

5 Pipet 1 mL into each well of poly-D-lysine coated 12-well plate

Day 2

6 Add 250 mL of 5x doxycycline for all desired concentrations to cells 14 hours before assay

Day 3

7 Prepare > 300 uL ligand-containing media per well (e.g. 10 uM AngII) for stimulation

8 Remove media from plate wells by vacuum in cell culture hood

9 Add 300 uL ligand-containing media per well and return to incubator for 1 hour

10 Prepare saturating concentration of membrane-impermeable radioligand in media on ice. A ligand with high specific activity is preferred. For example, 30 uL 3H-AngII in 12 mL media. Prepare at least 4 mL per plate. Vortex briefly to mix

11 Take 1/3 of media with radioligand and add high concentration (orders of magnitude above radioligand concentration) of nonlabelled antagonist (e.g. 200 uM candesartan final concentration) for nonspecific binding measurement. Vortex briefly to mix

12 After 1 hour ligand incubation, place plates on ice and remove media by vacuum. Wash twice with 1 mL ice-cold PBS with $MgCl_2$ and $CaCl_2$ per well.

13 Strip ligand for 5 minutes with 2 mL acid wash buffer (0.2 M acetic acid, 0.5 M NaCl, pH 2.5) per well

14 Remove acid wash buffer by vacuum and wash 2-3 times with 1 mL ice-cold PBS with $MgCl_2$ and $CaCl_2$ per well.

15 Remove wash by vacuum. Add 300 uL media with radioligand per well on ice. Use top two rows of plate for total binding duplicates and bottom row for nonspecific binding. Wait ≥ 1.5 hours on ice

16 Pre-chill 50 mL per plate PBS with $MgCl_2$ and $CaCl_2$ on ice and pre-warm 0.1% SDS + 0.5 N NaOH in 42 °C water bath

17 Remove radioligand media into dedicated radioactive liquid waste vial using P1000 on ice. Remove nonspecific binding media last

18 Wash wells with 1 mL cold PBS with MgCl2 and CaCl2 on ice and collect using P1000 into radioactive liquid waste vial. Wash 4 times total. Check plates under microscope after last wash

19 Remove plates from ice and add 500 uL warm 0.1% SDS + 0.5 N NaOH per well. If there is precipitate in the solution, allow it to warm for longer. After adding, shake for ≥ 15 minutes

20 Collect samples from wells using P1000 and add to labeled scintillation vials. Add 300 uL of each radioligand media mix to two scintillation vials each for total counts. Add 5 mL scintillation cocktail (Lefko-Fluor) to each vial. Cap vials

21 Wipe vials with moist KimWipe and place in racks for scintillation counter. Place racks in counter and count using 3h_cpm_3_min protocol

22 Perform second count using different protocol flag on next day

Analysis

23 Copy binding counts into Excel spreadsheet

24 Calculate nonspecific counts for each receptor concentration

25 Calculate specific counts for each condition by averaging duplicates and subtracting nonspecific count

26 Calculate percent internalization as percent reduction in specific counts for ligand-stimulated condition compared to control