HaloTag autophagy flux assay

In 1 collection

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ABSTRACT

Pule-chaseable method for quantifying autophagy flux

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Day 1: Seeding plates

1 Seed 50,000 HeLa cells in each chamber of a 12-well treated tissue culture plate. Maintain cells in DMEM + 10% FBS + 1x Pen-strep.

2 Grow for 2 days until cells are ~80% confluent

Day 3: Transient Transfection

3 If cells do not need to be transfected, skip to “day 5”

4 Wash cells into DMEM + 10% FBS without antibiotic prior to transfection.

5 Prepare reagents for lipofection with lipofectamine 3000. Each well requires 1.2 µg of pmCherry vector or construct. Prepare a master mix consisting of 1.2 µg of DNA, 125 µL of opti-mem low serum medium, and 2.4 µL of P3000 reagent per well. Prepare an additional mix consisting of 125 µL of opti-mem and 2.4 µL of lipofectamine 3000 per well.

6 Combine the DNA + P3000 and lipofectamine solutions together, and allow to incubate for 10 minutes at room temperature.

7 After 10 min, dispense 250 µL of combined DNA/P3000/lipofectamine mixture into each well to be transfected.

8 Return plates to incubator, and incubate for two days before conducting experiment

Day 4: Swap medium

9 Swap medium of transfected cells with fresh DMEM + 10% FBS + Pen-strep

Day 5: Autophagy Induction and Cell Collection

10 Replace growth medium with labeling medium consisting of growth medium + 100 nM Janelia Fluor 549-conjugated HaloLigand.

11 Incubate at 37 C for 20 min.

12 Aspirate off labeling medium and wash with PBS or growth medium.

13 Wash cells into either growth medium + DMSO, growth medium + 10 µM Oligomycin/ 5 µM Antimycin, or Earle's Balanced Salt solution to start the experiment.

14 Return cells to incubator for either 3 H (for starvation autophagy measurements via EBSS) or 6 H (for mitochondrial depolarization via OA).

15 Collect cells via scraping into ice-cold PBS.

16 Pellet cells via tabletop centrifuge, aspirate off supernatant, and snap freeze in liquid nitrogen. Store at -80 C until next day.

Day 6: HaloTag gel shift measurement and quantification

17 Lyse cells via resuspension in 30 µL lysis buffer and 30 minute incubation at 4 C (50 mM HEPES pH 7.4, 150 mM NaCl, 0.5% NP-40, 1 mM TCEP, cOmplete protease inhibition tablet (Roche).

18 Measure protein concentration via a 96-well plate BCA assay. Briefly, pipette 200 µL of reconstituted BCA reagent (Thermo Fisher) in each well of a 96-well plate. Include 5 additional wells for a 3 mg/mL, 2 mg/mL, 1 mg/mL, PBS blank, and Lysis buffer blank. Into each 200 µL well, pipette 1 µL of sample, standard, or blank. Cover plate and incubate at 37 C for 30 min before measuring the absorbance of each well at 560 nm. If protein concentration is especially high, it might be necessary to make a dilute sample of each well sample prior to performing BCA assay.

19 In excel, subtract blank readings from measured absorbances, and plot a standard curve using the BSA standards. Using the standard curve, determine the concentration of each protein samples, and the volume necessary to load 20 µg each.

20 Prepare samples for SDS-PAGE via addition of loading buffer and 25 mM TCEP. Heat samples at 95 C for 2 min before loading onto gel. Omit a ladder.

21 Run gel at 120 V until the dye band has reached the bottom of the gel. Gently cut off dye band at the bottom of the gel, and transfer the gel to a clean container. Rinse in deionized water before imaging on a ChemiDoc using the AlexaFluor 548 channel. Use AutoExpose protocol unless trying to image particularly faint bands.

22 Open image file in Fiji, define rectangular selection that encompasses the first lane, and use the Gel Analyzer tool to highlight each lane before plotting the trace.

23 In the trace window, define a baseline, and then measure the area under each section of the curve that describes the band of interest. The highest MW band should represent the Su9-halo or the LC3-halo reporter, while the lowest MW band should represent processed halo.

24 Calculate the fraction of total Halo signal constituted by the lowest band over the total halo signal to quantify autophagy flux.