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SPION-mediated Lysosome Purification – RAW264.7

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We use this protocol and it's working

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Abstract

This protocol details steps taken to isolate lysosomes in RAW 264.7 cells.

Materials

DMEM (Thermo Fisher Scientific, 11965-092)
FBS (Thermo Fisher Scientific, 16140-071)
Penicillin/Streptomycin (Thermo Fisher Scientific, 15140122)
CellStripper (Corning, 356230)
PBS [1.1 mM KH2PO4 (J.T. Baker, 3246-01), 155.2 mM NaCl (Sigma-Aldrich, 3624-05), and 3 mM Na2HPO4 (J.T. Baker, 3828-05)]
SPION Particles (see https://www.protocols.io/view/synthesis-of-colloidal-dextran-conjugated-superpar-eq2lyn69pvx9/v1)
5 mM HEPES (pH 7.4, Thermo Fisher Scientific, 15630-080)
HB buffer (5 mM Tris Base (American Bio, AB02000-05000), 250 mM Sucrose (Sigma-Aldrich, S0389), and 1 mM EGTA pH 7.4, Sigma-Aldrich, E4378) supplemented with inhibitors [cOmplete mini EDTA-free protease inhibitor (Roche, 11836170001) and PhosSTOP (Roche, 4906837001)]
Dounce Homogenizers (DWK Life Sciences WheatonTM Dounce Tissue grinders, 06-434)
LS columns (Miltenyi Biotec, 130042401)
QuadroMACS separator (Miltenyi Biotec, 130-091-051)

Day 0

1 Split a ~90% confluent 15-cm dish of RAW 264.7 cells into 8 10-cm dishes.

1.1 4 10-cm dishes are sufficient for one condition (for example, WT DMSO treatment).

Day 1 3h 40m

2 Replace the media in each dish with DMEM containing FBS and P/S supplemented with 5 mM HEPES and 5% SPION particles for 01:00:00 . 1h

3 Wash each dish 2X with PBS.

4 Add fresh media (DMEM containing FBS and P/S) to wash out the SPIONs for 02:00:00 . 2h

4.1 At this point, any drug treatment can be added.

5 Wash each dish 2X with cold PBS on ice. Scrape each dish with 1 mL of PBS and combine each condition into a 15-mL Falcon tube.

5.1 For example, all 4 10-cm dishes from WT DMSO condition into 1 Falcon tube.

6 Centrifuge 300 rcf, 4°C, 00:05:00 each 15-mL Falcon tube. 5m

7 Remove the supernatant and resuspend the pellet in 1 mL ice-cold HB buffer for 00:05:00 on ice. 5m

7.1 HB buffer: 5 mM Tris Base, 250 mM Sucrose, and 1 mM EGTA pH 7.4 supplemented with inhibitors (cOmplete mini EDTA-free protease inhibitor and PhosSTOP).

8 Transfer the lysate to a Dounce homogenizer and homogenize 50 times each sample with the tight pestle on ice.

9 Centrifuge 800 rcf, 4°C, 00:10:00 the lysate to obtain the cell lysate. 10m

9.1 At this step, a portion of the supernatant cell lysate was kept for the “Cell Lysate”.

10 Wash LC columns once with 2.5 mL of HB buffer on a QuadroMACS separator.

11 Apply the remainder of the supernatant/cell lysate to the LC columns. Collect the flow-through and reapply it to the column.

12 Wash LC columns with 3 mL of HB buffer on a QuadroMACS separator.

13 Remove LC columns from the QuadroMACS separator and elute lysosomes inao ultracentrifuge tubes with 2.5 mL of HB buffer.

14 Ultracentrifuge 55000 rpm, 4°C, 00:20:00 using a TLA-100.3 rotor in a Beckman-Coulter Ultracentrifuge Max Optima. 20m

15 Remove the supernatant and resuspend the lysosome pellet in 50 µL of HB buffer and freeze at -20C.

Day 2+

16 Quantify protein concentrations using Coomassie Plus Protein Assay Reagent as per the manufacturer’s protocol. Prepare lysates for immunoblotting as in the Protein Lysate and Immunoblotting protocol.io.

Protocol references

This protocol was adapted from Rofe and Pryor, 2016 (DOI: 10.1101/pdb.prot084822).