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Endo-IP and Lyso-IP in hESCs and iNeurons

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Disclaimer

Abstract

Building off of our previously published EEA1-positive endosome isolation technique (Endo-IP), we describe an approach for capturing endosomes and TMEM192-positive lysosomes (Lyso-IP) from human embryonic stem cells (hESCs) and induced cortical-like neurons (iNeurons) providing a relatively rapid means by which to examine the endolysosomal system. This approach enriches hundreds of resident endolysosomal proteins and cargo, and enables downstream proteomic analysis of the endoslysomal system, along with other applications.

Endosomal immunoprecipitation (Endo-IP) from hESCs and iNeurons for immunoblotting and proteomics

1 Seed cells on 3×15-cm Matrigel-coated dishes per replicate.

Note

Note that untagged control cells must be included in each experiment to assess the IP efficiency.

1.1 For hESCs, seed cells 1-3 days before performing the Endo-IP such that they will be ~70% confluent on the day of the Endo-IP.

1.2 For iNeurons, seed cells on day 4, 5, or 6 of the differentiation, aiming for ~60% confluency, and perform the Endo-IP on day 21 of the differentiation.

Note

Endo-IP can be performed at any desired stage of differentiation.

2 Harvest cells.

2.1 Place each 15-cm on ice, gently pour media into a waste container, and gently aspirate remaining media.

2.2 Wash once on-plate by gently adding 2 mL ice-cold PBS then gently aspirating PBS.

2.3 Add 1 mL ice-cold PBS supplemented with protease inhibitors (Roche), gently detach cells with a cell scraper, and transfer cells to tubes on ice using a wide-bore transfer pipette.

2.4 To recover residual cells, add an additional 1 mL of ice-cold PBS supplemented with protease inhibitors to each plate, and transfer cells to corresponding tubes using a wide-bore transfer pipette.

2.5 Pellet cells at 500xg for 00:04:00 at 4 °C . Remove supernatant. 4m

2.6 Gently resuspend cells using a wide-bore transfer pipette with 950 µL KPBS (100mM potassium phosphate, 25mM KCl, pH 7.2) with protease inhibitors (KPBS+i).

3 Lyse cells.

3.1 Lyse cells on ice with 30 strokes of a 2mL Dounce homogenizer (DWK Life Sciences) and B type pestle.

Note

Cell lysis is a critical step that can affect the efficiency of the Endo-IP. Dounce cells gently but thoroughly, and ensure that bubbles do not form during douncing. Cell lysis efficiency can be gauged under a light microscope using trypan blue. Lysis of ~50-70% of the cells is ideal as higher percent lysed usually results in lower efficiency enrichment of EEA1-positive endosomes, possibly due to rupture of organelle membranes.

3.2 Pellet lysate at 1,000xg for 00:05:00 at 4 °C . Transfer supernatant to new tube on ice using wide-bore transfer pipette, and spin lysate again at 1,000xg for 00:05:00 at 4 °C . 10m

3.3 Transfer final post-nuclear supernatant (PNS) to a new tube on ice using a wide-bore transfer pipette, and estimate the total final volume of PNS.

3.4 Quantify total protein in all PNS samples using a Bradford protein assay or similar. Normalize samples based on protein quantification result such that each sample has the same total protein and same final volume, using KPBS+i to normalize volume.

3.5 For later immunoblotting, set aside 10 µL of each PNS and combine with 30 µL 1xRIPA buffer or similar and 10 µL 5xLDS buffer with DTT.

4 Endo-IP.

4.1 Prepare 70 µL anti-FLAG M2 magnetic bead slurry (Sigma-Aldrich) per sample, plus extra to account for a small amount of loss during washes. Wash beads three times with KPBS+i using a magnetic stand and resuspend in x µL KPBS+i, where x = (0.75)* (total original volume of slurry transferred from stock bottle of beads).

4.2 Incubate each normalized PNS sample with 70 µL washed anti-FLAG M2 magnetic bead slurry at 4 °C for 00:50:00 with gentle rotation. 50m

5 Wash beads & Elute.

5.1 After incubation, briefly (~ 00:00:01) spin down tube, place tube on magnetic rack, and set aside 10 µL of the flow through and combine with 30 µL 1xRIPA buffer or similar and 10 µL 5xLDS buffer with DTT for later immunoblotting. 1s

5.2 Remove the remainder of the flow through, and wash beads three times with 1000 µL KPBS+i.

Note

To ensure optimal enrichment and minimal background, washes must be performed quickly and thoroughly. Ensure beads stuck in the lid after incubation are washed along with the beads in the rest of the tube. To reduce background, sample can be transferred to a fresh tube on the final wash and/or protein low-bind tubes can be used starting at douncing step through final elution step.

5.3 Elute each sample with 120 µL 0.5% NP-40 in KPBS+i at 4 °C for 00:30:00 with gentle rotation. 30m

5.4 Briefly (~ 00:00:01) spin down tube after elution incubation, place tube on magnetic rack, and set aside 20 µL of eluate and combine with 6 µL 5xLDS and DTT for later immunoblotting. The remaining 100 µL of eluate can be further processed for proteomics or stored at -80 °C . 1s

Lysosomal immunoprecipitation (Lyso-IP) from hESCs and iNeurons for immunoblotting and proteomics

4m

6 Seed cells on 2×15-cm Matrigel-coated dishes per replicate.

Note

Note that untagged control cells must be included in each experiment to assess the IP efficiency.

6.1 For hESCs, seed cells 1-3 days before performing the Lyso-IP such that they will be ~70% confluent on the day of the Lyso-IP.

6.2 For iNeurons, seed cells on day 4, 5, or 6 of the differentiation, aiming for ~60% confluency, and perform the Lyso-IP on day 21 of the differentiation.

Note

Lyso-IP can be performed at any desired stage of differentiation.

7 Harvest cells.

7.1 Place each 15-cm on ice, gently pour media into a waste container, and gently aspirate remaining media.

7.2 Wash once on-plate by gently adding 2 mL ice-cold PBS then gently aspirating PBS.

7.3 Add 1 mL ice-cold PBS supplemented with protease inhibitors (Roche), gently detach cells with a cell scraper, and transfer cells to tubes on ice using a wide-bore transfer pipette.

7.4 To recover residual cells, add an additional 1 mL of ice-cold PBS supplemented with protease inhibitors to each plate, and transfer cells to corresponding tubes using a wide-bore transfer pipette.

7.5 Pellet cells at 500xg for 00:04:00 at 4 °C . Remove supernatant. 4m

7.6 Gently resuspend cells using a wide-bore transfer pipette with 950 µL KPBS (100mM potassium phosphate, 25mM KCl, pH 7.2) with protease inhibitors (KPBS+i).

8 Lyse cells.

8.1 Lyse cells on ice with 30 strokes of a 2mL Dounce homogenizer (DWK Life Sciences) and B type pestle.

Note

Cell lysis is a critical step that can affect the efficiency of the Lyso-IP. Dounce cells gently but thoroughly, and ensure that bubbles do not form during douncing. Cell lysis efficiency can be gauged under a light microscope using trypan blue. Lysis of ~50-70% of the cells is ideal as higher percent lysed usually results in lower efficiency enrichment of TMEM192-positive lysosomes possibly due to rupture of organelle membranes.

8.2 Pellet lysate at 1,000xg for 00:05:00 at 4 °C . Transfer supernatant to new tube on ice using wide-bore transfer pipette, and spin lysate again at 1,000xg for 00:05:00 at 4 °C . 10m

8.3 Transfer final post-nuclear supernatant (PNS) to a new tube on ice using a wide-bore transfer pipette, and estimate the total final volume of PNS.

8.4 Quantify total protein in all PNS samples using a Bradford protein assay or similar. Normalize samples based on protein quantification result such that each sample has the same total protein and same final volume, using KPBS+i to normalize volume.

8.5 For later immunoblotting, set aside 10 µL of each PNS and combine with 30 µL 1xRIPA buffer or similar and 10 µL 5xLDS buffer with DTT.

9 Lyso-IP.

9.1 Prepare 65 µL anti-HA magnetic bead slurry (Pierce) per sample, plus extra to account for a small amount of loss during washes. Wash beads three times with KPBS+i using a magnetic stand and resuspend in y µL KPBS+i, where y = total original volume of slurry transferred from stock bottle of beads.

9.2 Incubate each normalized PNS sample with 65 µL washed anti-HA magnetic bead slurry at 4 °C for 00:30:00 with gentle rotation. 30m

10 Wash beads & Elute.

10.1 After incubation, briefly (~ 00:00:01) spin down tube, place tube on magnetic rack, and set aside 10 µL of the flow through and combine with 30 µL 1xRIPA buffer or similar and 10 µL 5xLDS buffer with DTT for later immunoblotting. 1s

10.2 Remove the remainder of the flow through, and wash beads twice with 1000 µL high salt KPBS+i (supplemented with 150mM NaCl) and wash once with 1000 µL KPBS+i.

Note

To ensure optimal enrichment and minimal background, washes must be performed quickly and thoroughly. Ensure beads stuck in the lid after incubation are washed along with the beads in the rest of the tube. To reduce background, sample can be transferred to a fresh tube on the final wash and/or protein low-bind tubes can be used starting at douncing step through final elution step.

10.3 Elute each sample with 120 µL 0.5% NP-40 in KPBS+i at 4 °C for 00:30:00 with gentle rotation. 30m

10.4 Briefly (~ 00:00:01) spin down tube after elution incubation, place tube on magnetic rack, and set aside 20 µL of eluate and combine with 6 µL 5xLDS and DTT for later immunoblotting. The remaining 100 µL of eluate can be further processed for proteomics or stored at -80 °C 1s

Protocol references

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