**Organelle isolation from Mouse Embryonic Fibroblasts (MEFs) stably expressing organelle tags for subsequent immunoblotting or proteomic analysis**

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**Abstract**

We describe here a method to perform rapid isolation of intact organelles (including lysosomes and Golgi) from mouse embryonic fibroblasts stably expressing an organelle tag (TMEM192-3xHA, or LysoTag, and TMEM115-3xHA, or GolgiTag). First, cells are broken using a ball-bearing cell breaker, leading to plasma membrane rupture, while lysosomes and Golgi remain intact. Then, the cell homogenate is incubated with anti-HA magnetic beads to allow for immunopurification of HA-tagged lysosomes or Golgi in less than 15 minutes. The organelles purified using this method are highly enriched, intact, contaminant-free and, depending on solubilisation buffer, can be used for various downstream applications, including immunoblotting analysis and mass spectrometry analysis (as described here), but also metabolomic or lipidomic analysis. This protocol can be adapted to isolate organelles from commonly cultured cells, such as HEK293 and A549 cells, that express an organelle tag.

*For the generation of mouse embryonic fibroblasts stably expressing organelle tags, refer to* protocol to be submitted.

**Materials**

1. **Reagents**

* MEFs stably expressing organelle tag: GolgiTag (Tmem115-3xHA) or LysoTag (Tmem192-3xHA), or control empty tag (3xHA).

*For the generation of MEFs stably expressing organelle tags, see protocol …*

* Dulbecco's phosphate-buffered saline (PBS) (GIBCO. REF# 14190169)
* KPBS Buffer: 136mM KCL, 10 mM KH2PO4 in MS grade water. Adjust to pH 7.25 with KOH.
  + - “Supplemented KPBS” (to be prepared immediately before harvesting the cells): KPBS buffer supplemented with 1X phosSTOP phosphatase inhibitor cocktail (PhosSTOP tablet: Roche, REF# 04906837001) and 1X protease inhibitor cocktail (cOmplete EDTA-free protease inhibitor cocktail tablet: Roche, REF# 11873580001)
* Thermo Scientific™ Pierce™ Anti-HA Magnetic Beads (Thermo Fisher Scientific, cat # 13474229)
* Lysis Buffer for Immunoblotting analysis: 50 mM Tris-HCl, pH 7.5, 1% (by volume) Triton X-100, 10% (by volume) glycerol, 150 mM NaCl, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 0.1 µg/ml microcystin-LR, and 1 tablet of cOmplete Mini (EDTA-free) protease inhibitor (Roche)
* Lysis Buffer for Mass spectrometry analysis: 2% v/v SDS, 20 mM HEPES pH 8, 1X phosSTOP phosphatase inhibitor cocktail (Roche), 1X protease inhibitor cocktail (complete EDTA-free, Roche)
* STrap washing buffer: 90% MeOH, 10% TEABC at pH 7.2
* LC-MS grade H2O (Thermo Fisher Scientific; Cat# 10777404)
* TEABC (Sigma; Cat# 18597) – Make a 50 mM and 300 mM stock in LC-MS grade H2O, pH 8
* TCEP (Thermo Fisher Scientific; Cat# 20491) – Make a 100 mM stock in 300 mM TEABC
* Iodoacetamide (IAA) (Sigma - I1149) – Make a 200 mM stock in 300 mM TEABC
* Trypsin/Lys-C Mix (Mass Spec Grade; Promega, UK, Cat# V5073) – 20 µg of trypsin/Lys-C reconstitute in 800 µL of 50 mM TEABC at a final concentration of 25 µg/mL
* Sodium dodecyl sulfate (SDS) micropellets (Formedium, Cat# SDS0500) – make a 20% solution in MilliQ water
* Phosphoric acid (Sigma; Cat# 345245) – Make a 12.5% solution in MilliQ water
* LC-MS grade Methanol (MeOH) (VWR; Cat# 20847.307)
* LC-MS grade Acetonitrile (ACN) (VWR; Cat# 83640.320)
* Formic acid (Sigma; Cat # 56302)
* Trifluoracetic acid (TFA) (Sigma; Cat# T6508)

1. **Equipment**

* Belly Dancer Orbital Shaker (IBI Scientific, model # BDRAA115S)
* DynaMag™-2 Magnet (Invitrogen. REF# 12321D)
* Isobiotec Cell-Breaker (isobiotec Vertriebs UG)
* Microcentrifuge with thermostat (VWR Micro Star 17R. S/N 42209232. REF# 521-1647)
* Bioruptor (Diagenode)
* Thermomixer (Eppendorf, UK)
* SpeedVac Vacuum Concentrator
* UltiMate 3000 RSLC nano-HPLC system (Thermo Fisher Scientific, UK) coupled to an Orbitrap ExplorisTM 480 mass spectrometer (Thermo Fisher Scientific, UK)
* Precolumn: Acclaim PepMapTM 100, C18, 100 µm x 2 cm, 5 µm, 100 Å
* Analytical column: PepMapTM RSLC C18, 75 µm x 50 cm, 2 µm, 100 Å

1. **Consumables**

* Disposable cell lifters (Sigma-Aldrich CLS3008)
* 1.5 ml low binding Eppendorf tubes (Sarstedt. REF# 72.706.600).
* Standard 1 ml and 200 µl Pipette tips (Greiner bio-one. Cat# 686271 and 685261 respectively).
* Stripetter/stripette gun and stripettes
* Set of Gilson pipettes P10, P200, P1000
* 1 ml Terumo Syringe without needle (Terumo REF# MDSS01SE)
* BD Microlance 3, 21G needle (Becton Dickinson. REF# 304432)
* Syringe without needle (5 ml) (Sigma-Aldrich. REF# Z116866)
* S-TrapTM columns (ProtiFi, USA, Cat# C02-micro)

**Steps:**

1. **Isobiotec cell-breaker assembly:**
   1. Insert the metal ball of choice inside the cell breaker.

*Note: For MEFs, we recommend a 12 μm clearance.*

* 1. Screw the lids on tightly.
  2. Push 3 ml of KPBS through the cell breaker to wash it.
  3. Carefully tap dry.
  4. Place the cell-breaker on aluminium foil on ice until use (Step 3.12).

**Note: To clean the Isobiotec cell-breaker between samples and at the end of the experiment:**

* Open the cell-breaker from one side.
* Take the metal ball out and rinse with MillIQ water.
* Flush the cell breaker thoroughly with MilliQ water using 5 mL syringes through both syringe inlets whilst covering the opening on the side of the cell breaker.
* Reassemble the cell-breaker by re-inserting the metal ball into the instrument and close the side panel tightly using the screws.
* Flush the cell breaker through both syringe inlets with 5 ml of KPBS using 5 ml syringes.

*Note: There will be some residual KPBS left in the cell-breaker (approximately 200 µl), this is optimal.*

* Proceed to homogenise the next sample.
* Once finished, flush the cell breaker thoroughly with MilliQ water using 5 mL syringes through both syringe inlets whilst covering the opening on the side of the cell breaker.
* Take all pieces apart (both side panels, panel screws and the metal ball).
* Clean each part with a generous amount of 70% (v/v) ethanol in MilliQ water.
* Wipe all parts dry and leave pieces apart to air-dry overnight.

*Note: Packing up the cell-breaker before it is dry will lead to development of rust and colouring of the metal parts.*

1. **Anti-HA Magnetic beads preparation:**

2.1) Transfer n x 100 µl of anti-HA Magnetic Beads (where n = number of samples) into a low binding Eppendorf tube.

2.2) Immobilize the beads by placing the tube into a Dyna-Mag tube holder for 30s.

2.3) Remove the supernatant using a pipette.

2.4) Gently resuspend the beads in 1ml of KPBS.

2.5) Repeat steps 2.2 to 2.4.

2.6) Immobilize the beads by placing the tube into a Dyna-Mag tube holder for 30s.

2.7) Remove the supernatant using a pipette.

2.8) Gently resuspend the beads from step 2.7 in n x 100 µl of KPBS (where n = number of samples you have) to make a 1:1 slurry.

2.9) Aliquot the washed beads from step 2.8 into fresh low-binding Eppendorf tubes (100 μl of slurry for each sample).

2.10) Leave the tubes on ice until use (step 3.18).

1. **Organelle isolation from cells:**

3.1) For each experimental condition, seed cells into one 15 cm dish.

*Note: In parallel, seed cells transduced to express HA-empty as a control.*

* 1. When cells have reached a confluency of ~ 90%, aspirate the culture medium.
  2. Quickly wash once by adding 5 mL of PBS at room temperature.
  3. Completely aspirate the PBS.
  4. Add 1 mL of ice-cold supplemented KPBS.
  5. Place the cell dishes on ice.
  6. Scrape the cells on the dish using a cell lifter to ensure all cells are detached from the dish.
  7. Using a pipette, transfer the cell suspension to a low binding Eppendorf on ice.
  8. Spin down at 1000 g for 2 minutes at 4°C.
  9. Discard the supernatant.
  10. Resuspend the pellet in 1 mL of ice-cold supplemented KPBS.
  11. Using a 1 ml syringe and 21G needle, transfer the cell suspension from step 3.11 into a KPBS rinsed, ice-cold Isobiotec cell-breaker (with gap-size of 12 μm) kept on ice (Step 1.5).
  12. Homogenise the cells with 10 passes through the cell breaker using 2 x 1ml syringes.

*Note: One pass is defined by the cell suspension passing through both syringes.*

*The homogenisation requires more force with more passes. Pay extra care to make sure the syringes are securely in their seals and that the sample doesn’t leak out. If you encounter too much pressure for passing the homogenate through the cell-breaker, consider using a ball that leaves a larger clearance gap.*

* 1. Collect the homogenate from the cell breaker into a fresh Eppendorf tube using a 1ml syringe.

*Note: To extract as much sample as possible from the cell-breaker post-homogenisation, push air into the cell-breaker using a syringe and collect from the other seal using another syringe.*

* 1. Transfer the resulting homogenate to a low binding Eppendorf on ice.
  2. Preclear the homogenate by centrifugation at 1000 g for 2 minutes at 4°C.
  3. For each sample, transfer 100 μL to a new low binding Eppendorf (= input) on ice.
  4. Add the remaining homogenate to 100 μl of the pre-washed HA-Magnetic beads (Step 2.10).
  5. Mix gently by flicking the bottom of the tube.
  6. Incubate with agitation on a Belly Dancer orbital shaker for 5 minutes at 4°C.

*The following steps should ideally be performed in a 4°C cold room. If not available, then keep working on ice.*

* 1. Place the tubes from Step 3.20 on a magnetic tube holder for 30s to immobilise the beads.
  2. Discard the supernatant or collect as a flow-through sample.
  3. Resuspend the beads from Step 3.22 in 1 ml of supplemented KBPS.
  4. Immobilise the beads by placing the tubes in a Dyna-Mag tube holder for 30s.
  5. Discard the supernatant.
  6. Repeat steps 3.23 to 3.25 twice.
  7. Resuspend the beads in 1 ml of supplemented KPBS and transfer to a new low binding Eppendorf tube on ice.
  8. Place the tubes in a Dyna-Mag tube holder for 30s.
  9. Discard the supernatant.
  10. The organelle IP beads (from step 3.29) and the input (from step 3.17) can now be processed for either 1) immunoblotting analysis, or 2) mass spectrometry analysis.

1. **Sample analysis by immunoblotting:**

4.1) Input (from step 3.17):

* + 1. Dilute in Lysis Buffer compatible for Immunoblotting analysis to a 1:1 ratio.
    2. Incubate on ice for 10 minutes.
    3. Clarify by centrifugation at 17000 g 4°C for 10 minutes.
    4. Transfer the supernatant to a new low binding tube.
  1. Organelle IP beads (from step 3.29):
     1. Resuspend in 100 μl of lysis buffer compatible for immunoblot analysis.
     2. Incubate on ice for 10 minutes.
     3. Immobilise the beads by placing the tubes in a Dyna-Mag tube holder for 30s.
     4. Transfer the supernatant to a new low binding tube.
  2. Quantify protein concentration by BCA assay.
  3. Samples can be analysed by quantitative immunoblotting analysis as described in dx.doi.org/10.17504/protocols.io.bsgrnbv6, ensuring an equal protein amount of both the input and IP is loaded (~ 2 μg).

1. **Sample analysis by Mass Spectrometry:**
   1. **Sample Processing**
      1. Input (from step 3.17):

5.1.1.1) Dilute in lysis buffer compatible for mass spectrometry analysis to a 1:1 ratio.

5.1.1.2) Sonicate using a Bioruptor (30 seconds ON, 30 seconds OFF for 15 cycles).

5.1.1.3) Clarify by centrifugation at 17000 g for 10 minutes at 4°C.

5.1.1.4) Transfer the supernatant to a clean low binding tube.

* + 1. Organelle IP beads (from step 3.29):
       1. Resuspend in 100 μl of lysis buffer compatible for mass spectrometry analysis.
       2. Incubate at room temperature for 10 minutes.
       3. Sonicate using a Bioruptor (30 seconds ON, 30 seconds OFF for 15 cycles).
       4. Immobilise the beads by placing the tubes in a Dyna-Mag tube holder for 30s.
       5. Transfer the supernatant to a new low binding tube.
    2. Reduction: Add TCEP to the samples from step 5.1.1.4 and 5.1.2.5 to a final concentration of 5 mM and place on a thermomixer at 60°C with 1,100 rpm for 30 minutes.
    3. Cool the samples down to room temperature.
    4. Alkylation: Add IAA to the samples from step 5.1.4 to a final concentration of 20 mM and place on a thermomixer at 25°C with 1,100 rpm for 30 minutes, shielded from light.
    5. Add sodium dodecyl sulfate (SDS) to a final concentration of 5% (v/v) and phosphoric acid to a final concentration of 1.2% (v/v) to the samples from step 5.1.5.
    6. Dilute the sample with an additional volume of wash buffer (wash buffer volume equals to 6-fold of the sample volume) (90% MeOH, 10% TEABC at pH 7.2) and mix by vortexing.
    7. Load each sample onto a S-TrapTM column.
    8. Centrifuge at 1000 g for 1 minute.
    9. Discard the flow-through.
    10. Wash the S-TrapTM columns three times with 150 µL wash buffer (90% MeOH, 10% TEABC at pH 7.2). Discard the flowthrough after each wash.
    11. Transfer the S-Trap column to a fresh 1.5 mL low binding tube
    12. Prepare a Trypsin/Lys-C Mix in 50 mM TEABC solution, pH 8 to a 25 µg/mL concentration.
    13. On-column digestion: Add 60 µL (1.5 µg) Trypsin/Lys-C Mix from step 5.1.13 to each S-Trap column from step 5.1.11 and incubate on a thermomixer at 47°C for 1 hour with no agitation.
    14. Reduce the temperature on the thermomixer to 22°C and incubate overnight with no agitation.
    15. Peptide elution: Add 60 µL of 50 mM TEABC solution, pH 8 to each S-Trap column and centrifuge
    16. Add 60 µL of 0.15% (v/v) formic acid (FA) aqueous solution to each S-Trap column and centrifuge
    17. Add 60 µL of elution buffer (80% ACN with 0.15% FA in aqueous solution) to each S-Trap column and centrifuge
    18. Repeat step 5.1.18.
    19. Discard the S-Trap columns.
    20. Snap-freeze the samples on dry ice.
    21. Dry the samples at 35°C using a SpeedVac Vacuum Concentrator.
    22. Resuspend the samples from step 5.1.22 in 60 µL solution containing 3% (v/v) ACN and 0.1% (v/v) FA in LC-MS grade H2O
    23. Incubate the samples on a thermomixer at 22°C with 1200 rpm for 30 minutes.
    24. Sonicate the samples for 30-minutes in a water bath
    25. Estimate peptide concentration of each sample using a NanoDrop instrument by measuring the solution absorbance A280 at 224 nm wavelength.
  1. **Sample Injection onto Mass Spectrometer**

***Note: Liquid chromatography tandem mass spectrometry (LC-MS/MS) is performed using an UltiMate 3000 RSLC nano-HPLC system coupled to an Orbitrap ExplorisTM 480 mass spectrometer.***

* + 1. For each sample, load 4 µg of digested protein sample onto the nano-HPLC system individually.
    2. Trap the peptides using a precolumn (Acclaim PepMapTM 100, C18, 100 µm x 2 cm, 5 µm, 100 Å) using an aqueous solution containing 0.1% (v/v) TFA.
    3. Separate the peptides using an analytical column (PepMapTM RSLC C18, 75 µm x 50 cm, 2 µm, 100 Å) at 45°C using a linear gradient of 8 to 25% solvent B (an 80% ACN and 0.1% FA solution) for 98 minutes, 25 to 37% solvent B for 15 minutes, 37 to 95% solvent B for 2 minutes, 95% solvent B for 8.5 minutes, 95% to 3% solvent B for 0.5 minutes, and 3% solvent B for 9.5 minutes. Set the flow rate at 250 nL/min.
    4. Acquire data in data-independent acquisition (DIA) mode containing 45 isolated m/z windows ranging from 350 to 1500.
    5. Use a higher-energy collisional dissociation (HCD) with nitrogen for peptide fragmentation with the following isolation window:

|  |  |  |
| --- | --- | --- |
| **m/z** | **z** | **Isolation Window** |
| 383.4 | 3 | 66.8 |
| 423.0 | 3 | 13.5 |
| 435.0 | 3 | 11.5 |
| 446.5 | 3 | 12.5 |
| 458.0 | 3 | 11.5 |
| 469.0 | 3 | 11.5 |
| 480.0 | 3 | 11.5 |
| 490.5 | 3 | 10.5 |
| 501.0 | 3 | 11.5 |
| 512.0 | 3 | 11.5 |
| 523.0 | 3 | 11.5 |
| 533.5 | 3 | 10.5 |
| 544.0 | 3 | 11.5 |
| 554.5 | 3 | 10.5 |
| 565.0 | 3 | 11.5 |
| 575.5 | 3 | 10.5 |
| 586.0 | 3 | 11.5 |
| 597.5 | 3 | 12.5 |
| 609.5 | 3 | 12.5 |
| 621.5 | 3 | 12.5 |
| 633.0 | 3 | 11.5 |
| 645.0 | 3 | 13.5 |
| 657.5 | 3 | 12.5 |
| 670.5 | 3 | 14.5 |
| 684.0 | 3 | 13.5 |
| 697.0 | 3 | 13.5 |
| 710.5 | 3 | 14.5 |
| 725.5 | 3 | 16.5 |
| 741.0 | 3 | 15.5 |
| 756.5 | 3 | 16.5 |
| 773.5 | 3 | 18.5 |
| 791.0 | 3 | 17.5 |
| 808.5 | 3 | 18.5 |
| 827.0 | 3 | 19.5 |
| 846.5 | 3 | 20.5 |
| 866.5 | 3 | 20.5 |
| 887.5 | 3 | 22.5 |
| 910.5 | 3 | 24.5 |
| 935.5 | 3 | 26.5 |
| 962.5 | 3 | 28.5 |
| 992.0 | 3 | 31.5 |
| 1025.0 | 3 | 35.5 |
| 1063.0 | 3 | 41.5 |
| 1108.5 | 3 | 50.5 |
| 1391.6 | 3 | 516.8 |

* 1. **Data analysis**
     1. The DIA MS experiment's raw data were analysed using the DIA-NN software (Reference 1), employing a library-free search mode based on a reviewed Swiss-Prot database downloaded from UniProt.
     2. Trypsin/P was selected as the digestive enzyme, and up to 2 missed cleavages were allowed. Carbamidomethylation at Cysteine residue was set as a fixed modification, while oxidation at methionine residue was included as a variable modification. The software automatically detected and adjusted the mass error (ppm).
     3. A protein identification cut-off of 1% FDR was used, and a protein quantification required a minimum of 2 peptides in at least 75% samples.
     4. The protein group search results generated from DIA-NN software were then imported into Perseus software (Reference 2) for statistical analysis.
     5. For the organelle-IP samples, IP samples were first compared against the relevant mock IP samples to classify proteins significantly enriched, using a fold-change > 1.5 and p-value < 0.05.
     6. The organelle enriched proteins were then compared against genotypes or treatments to investigate protein level changes at the targeted organelle.
     7. For the whole cell lysate samples, proteins were directly compared against genotypes or treatments to determine the proteome changes in the cells.
     8. Significant up-/down-regulated proteins (fold-change > |1.5| and p-value < 0.05) obtained from organelle-IP and whole cell lysate samples were then submitted to metascape (reference 3) for enrichment analysis.
     9. The clustering analysis using metascape focuses on enrichment of GO biological processes pathway, GO molecular functions, and GO cellular components with p-value < 0.01
     10. The text files generated from Perseus software were imported into an in-house software, Curtain 2.0, for data visualisation.

References

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