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

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We use this protocol and it's working

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DISCLAIMER

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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 proteomic 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.

ATTACHMENTS

meh7bhc4x.docx

GUIDELINES

Protocol overview:

1. Organelle isolation from cells expressing organelle tags.
2. Analysis of isolated organelles by immunoblotting
3. Analysis of isolated organelles by mass spectrometry (proteomic analysis)

For the generation of MEFs stably expressing organelle tags, see ***dx.doi.org/10.17504/protocols.io.6qpvr456bgmk/v1***

MATERIALS

Reagents

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

Note

For the generation of MEFs stably expressing organelle tags, see **dx.doi.org/10.17504/protocols.io.6qpvr456bgmk/v1**

- DPBS no calcium no magnesium **Gibco - Thermo Fisher Catalog #14190169**

- **KPBS Buffer**:

	A	B
	KCL	136 mM
	KH_2PO_4 in MS grade water	10 mM

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**:

	A	B
	Tris-HCl, pH 7.5	50 mM
	Triton X-100	1% (by volume)
	glycerol	10% (by volume)
	NaCl	150 mM
	sodium orthovanadate	1 mM
	sodium fluoride	50 mM
	sodium β-glycerophosphate	10 mM
	sodium pyrophosphate	5 mM
	microcystin-LR	0.1 µg/ml
	cOmplete Mini (EDTA-free) protease inhibitor (Roche)	1 tablet

- **Lysis Buffer for Mass spectrometry analysis**:

	A	B
	SDS	2% v/v
	HEPES pH 8	20 mM
	phosSTOP phosphatase inhibitor cocktail (Roche)	1X

	A	B
	protease inhibitor cocktail (completeEDTA-free, Roche)	1X

- **STrap washing buffer**:

	A	B
	MeOH	90%
	TEABC at pH 7.2	10%

- LC-MS grade H_2O (LC-grade Water **Fisher Scientific Catalog #10777404**)
- Triethylammonium bicarbonate buffer **Merck MilliporeSigma (Sigma-Aldrich) Catalog #18597** – Make a [M] 50 millimolar (mM) and [M] 300 millimolar (mM) stock in LC-MS grade H_2O, pH 8
- Pierce™ TCEP-HCl **Thermo Fisher Catalog #20491** –Make a [M] 100 millimolar (mM) stock in [M] 300 millimolar (mM) TEABC
- Iodoacetamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #I1149** – Make a [M] 200 millimolar (mM) stock in [M] 300 millimolar (mM) TEABC
- Trypsin/Lys-C Mix, Mass Spec Grade, 5 x 20ug **Promega Catalog #V5073**) – 20 µg of trypsin/Lys-C reconstitute in 800 µL of [M] 50 millimolar (mM) TEABC at a final concentration of 25 µg/mL
- SDS Micro-Pellets **Formedium Catalog #SDS0500** – make a 20% solution in MilliQ water
- Phosphoric acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #345245** – Make a 12.5% solution in MilliQ water
- Methanol LC-MS grade B&J Brand **VWR International Catalog #BJLC230-2.5**
- Acetonitrile LC-MS grade B&J Brand **VWR International Catalog #BJLC015-2.5**
- Formic acid (Sigma; Cat # 56302)
- Trifluoroacetic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T6508**

Equipment

Equipment

BELLY DANCER ORBITAL SHAKER	NAME
ORBITAL SHAKER	TYPE
IBI	BRAND
BDRAA115S	SKU
https://www.ibisci.com/products/belly-dancer-shaker	LINK

Equipment

DynaMag-2	NAME
Magnet	TYPE
Invitrogen	BRAND
12321D	SKU
https://www.thermofisher.com/order/catalog/product/12321D#/12321D	LINK

- Isobiotec Cell-Breaker (isobiotec Vertriebs UG)

Equipment

Micro Star 17 / 17R, Microcentrifuges, Ventilated/Refrigerated	NAME
Microcentrifuge	TYPE
VWR®	BRAND
521-1647	SKU
https://in.vwr.com/store/product/8306728/microcentrifuges-ventilated-refrigerated-micro-star-17-17r	LINK

- 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 PepMap^{TM} 100, C18, 100 µm x 2 cm, 5 µm, 100 Å
- Analytical column: PepMap^{TM} RSLC C18, 75 µm x 50 cm, 2 µm, 100 Å

Consumables

- Corning® cell lifter **Merck MilliporeSigma (Sigma-Aldrich) Catalog #CLS3008 100EA**
- SafeSeal reaction tube 1.5 ml PP PCR Performance Tested Low protein-binding **Sarstedt Catalog #72.706.600**
- Greiner Bio-One™ Polypropylene Pipette Tip **Fisher Scientific Catalog #686271**

  and

  PIPETTE TIP 10 - 100 µL SUITABLE FOR EPPENDORF 96 PIECES / ST RACK **greiner bio-one Catalog #685261**
- Stripetter/stripette gun and stripettes
- Set of Gilson pipettes P10, P200, P1000
- Terumo® Syringe 3-part Syringe **Terumo Catalog #MDSS01SE**
- Becton Dickinson Disposable needles 21G x 1 1/2 inch **Becton-Dickinson Catalog #304432**
- Syringe PP/PE without needle **Merck MilliporeSigma (Sigma-Aldrich) Catalog #Z116866**
- S-Trap™ micro columns (≤ 100 µg) **Protifi Catalog #C02-micro**

Isobiotec cell-breaker assembly

1 Insert the metal ball of choice inside the cell breaker.

Note

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

2 Screw the lids on tightly.

3 Push 3 mL of KPBS through the cell breaker to wash it.

4 Carefully tap dry.

5 Place the cell-breaker on aluminium foil On ice until use (Step 28).

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

6.1 Open the cell-breaker from one side.

6.2 Take the metal ball out and rinse with MilliQ water.

6.3 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.

6.4 Reassemble the cell-breaker by re-inserting the metal ball into the instrument and close the side panel tightly using the screws.

6.5 Flush the cell breaker through both syringe inlets with 5 mL of KPBS using 5-ml syringes.

Note

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

6.6 Proceed to homogenise the next sample.

6.7 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.

6.8 Take all pieces apart (both side panels, panel screws and the metal ball).

6.9 Clean each part with a generous amount of 70% (v/v) ethanol in MilliQ water.

6.10 Wipe all parts dry and leave pieces apart to air-dry Overnight.

Note

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

Anti-HA Magnetic beads preparation

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

8 Immobilize the beads by placing the tube into a Dyna-Mag tube holder for 00:00:30. 30s

9 Remove the supernatant using a pipette.

10 Gently resuspend the beads in 1 mL of KPBS.

11 Repeat steps 8 to 10.

12 Immobilize the beads by placing the tube into a Dyna-Mag tube holder for 00:00:30. 30s

13 Remove the supernatant using a pipette.

14 Gently resuspend the beads from step 13 in n x 100 µL of KPBS (where n = number of samples you have) to make a 1:1 slurry.

15 Aliquot the washed beads from step 14 into fresh low-binding Eppendorf tubes (100 µL of slurry for each sample).

16 Leave the tubes On ice until use (step 34).

Organelle isolation from cells expressing organelle tags

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

Note

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

18 When cells have reached a confluency of ~ 90%, aspirate the culture medium.

19 Quickly wash once by adding 5 mL of PBS at Room temperature .

20 Completely aspirate the PBS.

21 Add 1 mL of ice-cold supplemented KPBS.

22 Place the cell dishes On ice.

23 Scrape the cells on the dish using a cell lifter to ensure all cells are detached from the dish.

24 Using a pipette, transfer the cell suspension to a low binding Eppendorf On ice.

25 Spin down at 1000 x g, 4°C, 00:02:00. 2m

26 Discard the supernatant.

27 Resuspend the pellet in 1 mL of ice-cold supplemented KPBS.

28 Using a 1-ml syringe and 21G needle, transfer the cell suspension from step 27 into a KPBS rinsed, ice-cold Isobiotec cell-breaker (with gap-size of 12 µm) kept On ice (Step 5).

29 Homogenise the cells with 10 passes through the cell breaker using 2 x 1-ml syringes.

Note

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.

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

Note

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.

31 Transfer the resulting homogenate to a low binding Eppendorf On ice.

32 Preclear the homogenate by centrifugation at 1000 x g, 4°C, 00:02:00. 2m

33 For each sample, transfer 100 µL to a new low binding Eppendorf (= input) On ice.

34 Add the remaining homogenate to 100 µL of the pre-washed HA-Magnetic beads (Step 16).

35 Mix gently by flicking the bottom of the tube.

36 Incubate with agitation on a Belly Dancer orbital shaker for 00:05:00 at 4 °C. 5m

37 30s

Note

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

Place the tubes from Step 36 on a magnetic tube holder for 00:00:30 to immobilise the beads.

38 Discard the supernatant or collect as a flow-through sample.

39 Resuspend the beads from Step 38 in 1 mL of supplemented KBPS.

40 Immobilise the beads by placing the tubes in a Dyna-Mag tube holder for 00:00:30. 30s

41 Discard the supernatant.

42 Repeat steps 39 to 41 twice.

43 Resuspend the beads in 1 mL of supplemented KPBS and transfer to a new low binding Eppendorf tube On ice.

44 Place the tubes in a Dyna-Mag tube holder for 00:00:30. 30s

45 Discard the supernatant.

46 The organelle IP beads (from step 45) and the input (from step 33) can now be processed for either 1) immunoblotting analysis, or 2) mass spectrometry analysis, as detailed below..

Sample analysis by immunoblotting

47 **Input** (from step 33)

47.1 Dilute in Lysis Buffer compatible for Immunoblotting analysis to a 1:1 ratio.

47.2 Incubate On ice for 00:10:00. 10m

47.3 Clarify by centrifugation at 17000 x g, 4°C, 00:10:00. 10m

47.4 Transfer the supernatant to a new low binding tube.

48 **Organelle IP beads** (from step 45).

48.1 Resuspend in 100 µL of lysis buffer compatible for immunoblot analysis.

48.2 Incubate On ice for 00:10:00. 10m

48.3 Immobilise the beads by placing the tubes in a Dyna-Mag tube holder for 00:00:30. 30s

48.4 Transfer the supernatant to a new low binding tube.

49 Quantify protein concentration by BCA assay.

50 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).

51 **Input** (from step 33):

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

51.2 Sonicate using a Bioruptor (00:00:30 ON, 00:00:30 OFF for 15 cycles). 1m

51.3  Clarify by centrifugation at 17000 x g, 4°C, 00:10:00 . 10m

51.4 Transfer the supernatant to a clean low binding tube.

52 **Organelle IP beads** (from step 45):

52.1 Resuspend in 100 µL of lysis buffer compatible for mass spectrometry analysis.

52.2  Incubate at Room temperature for 00:10:00 . 10m

52.3 Sonicate using a Bioruptor (00:00:30 ON, 00:00:30 OFF for 15 cycles). 1m

52.4 Immobilise the beads by placing the tubes in a Dyna-Mag tube holder for 00:00:30 . 30s

52.5 Transfer the supernatant to a new low binding tube.

53 **Reduction**: Add TCEP to the samples from step 51.4 and 52.5 to a final concentration of [M] 5 millimolar (mM) and place on a thermomixer at 1100 rpm, 60°C, 00:30:00 .

54 Cool the samples down to Room temperature .

55 **Alkylation**: Add IAA to the samples from step 54 to a final concentration of [M] 20 millimolar (mM) and place on a thermomixer at 1100 rpm, 25°C, 00:30:00 , shielded from light.

56 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 55.

57 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.

58 Load each sample onto a S-TrapTM column.

59 Centrifuge at 1000 x g, 00:01:00 . 1m

60 Discard the flow-through.

61 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.

62 Transfer the S-Trap column to a fresh 1.5-mL low binding tube.

63 Prepare a Trypsin/Lys-C Mix in [M] 50 millimolar (mM) TEABC solution, pH 8 to a 25 µg/mL concentration.

64 On-column digestion: Add 60 µL (1.5 µg) Trypsin/Lys-C Mix from step 63 to each S-Trap column from step 61 and incubate on a thermomixer at 47 °C for 01:00:00 with no agitation. 1h

65 Reduce the temperature on the thermomixer to 22 °C and incubate Overnight with no agitation. 1h

66 **Peptide elution**: Add 60 µL of 50 millimolar (mM) TEABC solution, pH 8 to each S-Trap column and centrifuge.

67 Add 60 µL of 0.15% (v/v) formic acid (FA) aqueous solution to each S-Trap column and centrifuge.

68 Add 60 µL of elution buffer (80% ACN with 0.15% FA in aqueous solution) to each S-Trap column and centrifuge.

69 Repeat step 68.

70 Discard the S-Trap columns.

71 Snap-freeze the samples on dry ice.

72 Dry the samples at 35 °C using a SpeedVac Vacuum Concentrator.

73 Resuspend the samples from step 72 in 60 µL solution containing 3% (v/v) ACN and 0.1% (v/v) FA in LC-MS grade H_2O.

74 Incubate the samples on a thermomixer at 1200 rpm, 22°C, 00:30:00.

75 Sonicate the samples for 00:30:00 in a water bath. 30m

76 Estimate peptide concentration of each sample using a NanoDrop instrument by measuring the solution absorbance A280 at 224 nm wavelength.

Sample analysis by Mass Spectrometry: Sample Injection o...

77

Note

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

For each sample, load 4 µg of digested protein sample onto the nano-HPLC system individually.

78 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.

79 Separate the peptides using an analytical column (PepMapTM RSLC C18, 75 µm x 50 cm, 2 µ 2h 13m 30s 100 Å) at 45 °C using

- a linear gradient of 8 to 25% solvent B (an 80% ACN and 0.1% FA solution) for 01:38:00,
- 25 to 37% solvent B for 00:15:00,
- 37 to 95% solvent B for 00:02:00,
- 95% solvent B for 00:08:30,

- 95% to 3% solvent B for 00:00:30, and
- 3% solvent B for 00:09:30.

Set the flow rate at 250 nL/min.

80 Acquire data in data-independent acquisition (DIA) mode containing 45 isolated m/z windows ranging from 350 to 1500.

81 Use a higher-energy collisional dissociation (HCD) with nitrogen for peptide fragmentation with the following isolation window:

	A	B	C
	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

	A	B	C
	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

Sample analysis by Mass Spectrometry: Data analysis

82 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.

83 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).

84 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.

85 The protein group search results generated from DIA-NN software were then imported into Perseus software (Reference 2) for statistical analysis.

86 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.

87 The organelle enriched proteins were then compared against genotypes or treatments to investigate protein level changes at the targeted organelle.

88 For the whole cell lysate samples, proteins were directly compared against genotypes or treatments to determine the proteome changes in the cells.

89 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.

90 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.

91 The text files generated from Perseus software were imported into an in-house software, Curtain 2.0, for data visualisation.