2

Apr 01, 2022

Endosomal and lysosomal immunoprecipitation for proteomics, lipidomics, and TEM V.2

Hankum Park[1,2,3], Frances V Hundley[1,2], J. Wade Harper[1,2]

[1]Department of Cell Biology, Harvard Medical School Boston, MA 02115, USA;
[2]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD 20 815, USA;
[3]Current affiliation: Seoul National University, School of Dentistry

1

dx.doi.org/10.17504/protocols.io.ewov14pjyvr2/v2

Frances Hundley

Previous studies have developed methods for isolation of lysosomes, mitochondria, and peroxisomes from non-denaturing extracts. Here we describe an approach for purification of early/sorting endosomes, providing a means by which to examine early aspects of the endolysosomal system and to combine this with lysosome purification using Lyso-IP. We refer to this new method as Endo-IP. This allows us to examine the proteome and lipidome of endosomes, and to perform electron microscopy imaging of endosomes.

DOI

dx.doi.org/10.17504/protocols.io.ewov14pjyvr2/v2

Citation: Hankum Park, Frances V Hundley, J. Wade Harper Endosomal and lysosomal immunoprecipitation for proteomics, lipidomics, and TEM https://dx.doi.org/10.17504/protocols.io.ewov14pjyvr2/v2

Hankum Park, Frances V Hundley, J. Wade Harper 2022. Endosomal and lysosomal immunoprecipitation for proteomics, lipidomics, and TEM. **protocols.io** https://dx.doi.org/10.17504/protocols.io.ewov14pjyvr2/v2 Frances Hundley

ASAPCRN

protocol ,

Mar 25, 2022

Apr 01, 2022

59926

A	B	C
REAGENT or RESOURCE	**SOURCE**	**IDENTIFIER**
Antibodies		
anti-EEA1 (C45B10) rabbit mAb	Cell Signaling Technology	3288
anti-RAB5 (C8B1) rabbit mAb	Cell Signaling Technology	3547
anti-PSEN1 (D39D1) rabbit mAb	Cell Signaling Technology	5643
anti-PSEN2/AD5 (EP1515Y) rabbit mAb	Abcam	ab51249
anti-LAMP1 (D2D11) rabbit mAb	Cell Signaling Technology	9091
anti-LAMP2 (D5C2P) rabbit mAb	Cell Signaling Technology	49067
anti-TMEM192 rabbit pAb	Proteintech	28263-1-AP
anti-HA	Biolegend	901513
anti-HA (6E2) mouse mAb	Cell Signaling Technology	2367
anti-FLAG M2 mouse mAb	Sigma-Aldrich	F1804
anti-ZO-1 rabbit pAb	Proteintech	21773-1-AP
anti-Golga1 rabbit pAb	Proteintech	12640-1-AP

anti-Calreticulin rabbit pAb	Proteintech	10292-1-AP
anti-S6K rabbit pAb	Proteintech	14485-1-AP
anti-RAB11 (D4F5) rabbit mAb	Cell Signaling Technology	5589
anti-Lamin A/C (4C11) mouse mAb	Cell Signaling Technology	4777
anti-VDAC1/Porin rabbit pAb	Proteintech	55259-1-AP
anti-RAB7 (D95F2) rabbit mAb	Proteintech	9367
anti-DYKDDDDK tag, mouse mAb (FG4R)	Thermo Fisher Scientific	MA1-91878
anti-GAPDH (D16H11) XP rabbit mAb	Cell Signaling Technology	5174
anti-APP CTF (C1/6.1) mouse mAb	BioLegend	802801
anti-APP A4 (22C11) mouse mAb	Sigma	MAB348
anti-PEX19 rabbit pAb	Proteintech	14713-1-AP
anti-CD71/TFR1 (D7G9X) rabbit mAb	Cell Signaling Technology	13113
anti-HSP90 (3F11C1) mouse mAb	Proteintech	60318-1-Ig
anti-BACE1 (D10E5) rabbit mAb	Cell Signaling Technology	5606
IRDye 680RD Goat anti-Rabbit IgG secondary antibody	Li-Cor	926-68071
IRDye 680RD Goat anti-Mouse IgG secondary antibody	Li-Cor	926-68070
IRDye 800CW Goat anti-Rabbit IgG secondary antibody	Li-Cor	926-32211
IRDye 800CW Goat anti-Mouse IgG secondary antibody	Li-Cor	926-32210
Goat anti-Rabbit IgG, HRP-linked antibody	Cell Signaling Technology	7474P2
Goat anti-Rabbit IgG HRP conjugate	Bio-Rad	1706515
Goat anti-Mouse IgG HRP conjugate	Bio-Rad	1706516
Chemicals, peptides, and recombinant proteins		
anti-FLAG M2 magnetic beads	Sigma-Aldrich	M8823
Pierce anti-HA magnetic beads	Thermo Fisher Scientific	88837

Citation: Hankum Park, Frances V Hundley, J. Wade Harper Endosomal and lysosomal immunoprecipitation for proteomics, lipidomics, and TEM https://dx.doi.org/10.17504/protocols.io.ewov14pjyvr2/v2

TMT10plex Isobaric Label Reagent Set plus TMT11-131C Label Reagent	Thermo Fisher Scientific	A34808
TMTProTM 16Plex Label Reagent set	Thermo Fisher Scientific	A44520
Super Heavy TMT Label Reagent	Thermo Fisher Scientific	A43073
Pierce™ High pH Reversed-Phase Peptide Fractionation Kit	Thermo Fisher Scientific	84868
HyClone Fetal bovine serum	GE Healthcare	SB30910
Puromycin	Sigma-Aldrich	P9620
G418 (Geneticin)	Invivogen	ant-gn-2
Dulbecco's MEM (DMEM), high glucose, pyruvate	GIBCO / Invitrogen	11995
PhosSTOP	Roche	04906845001
Complete EDTA-free protease inhibitor cocktail	Sigma-Aldrich	11873580001
Tris(2-carboxyethyl)phosphine hydrochloride solution	Sigma-Aldrich	646547
Iodoacetamide	Sigma-Aldrich	I1149
Trichloroacetic acid solution 6.1 N	Sigma-Aldrich	T0699
Trifluoroacetic acid	fisher scientific	A11650
Hydroxylamine solution 50 wt. %	Sigma-Aldrich	438227
Formic Acid	Sigma-Aldrich	5330020050
Pierce Trypsin Protease, MS grade	Thermo Fisher Scientific	90305
Lysyl endopeptidaseR (Lys-C)	Wako	129-02541
REVERT 700 total protein stain kit	Li-Cor	926-11016
NuPAGE LDS sample buffer (4X)	Thermo Fisher Scientific	NP0007
NuPAGE sample reducing agent (10X)	Thermo Fisher Scientific	NP0009
NuPAGE MES SDS Running Buffer (20X)	Thermo Fisher Scientific	NP0002
Immobilon-FL PVDF Membrane	Millipore	IPFL00010
WHEATON Dounce Tissue Grinder, 7 mL	DWK Life Sciences	357542

Citation: Hankum Park, Frances V Hundley, J. Wade Harper Endosomal and lysosomal immunoprecipitation for proteomics, lipidomics, and TEM https://dx.doi.org/10.17504/protocols.io.ewov14pjyvr2/v2

KIMBLE KONTES Dounce Tissue Grinder, 2 mL	DWK Life Sciences	885300-0002
Nonidet P40 substitute	Sigma-Aldrich	74385
Urea	Sigma-Aldrich	U5378
EPPS 0.2M buffer solution, pH 8.5	Alfa Aesar	J61476.AE
Empore C18 47 mm Extraction Disc, Model 2215	3M	98060402173
Sep-Pak C18 1 cc Vac Cartridge	Waters	WAT054955
Dyngo4a	Cayman Chemical	29479
Lanabecestat (AZD3293)	Selleckchem	S8193
Semagacestat	Cayman Chemical	16713
BPN-15606	MedChemExpress	HY-117482
RIPA lysis and extraction buffer	Thermo Fisher Scientific	89900
Reference peptides for APP/Ab (see Supplemental Data Table S7)	Biomatik Thermo Fisher Scientific	Custom order
Experimental models: Cell lines		
293 cells	ATCC	CRL-1573
293EL-APP-/-: TMEM192-3xHA; APP-/-; FLAG-EEA1	This study	
293EL-APP*: TMEM192-3xHA; APP-/-; FLAG-EEA1; APPSw;T700N	This study	

:

Citation: Hankum Park, Frances V Hundley, J. Wade Harper Endosomal and lysosomal immunoprecipitation for proteomics, lipidomics, and TEM https://dx.doi.org/10.17504/protocols.io.ewov14pjyvr2/v2

Lysosomal immunoprecipitation (Lyso-IP) for organelle proteomics

1 Seed 293 cells or 293^{EL} cells expressing TMEM192-3xHA and 3xFLAG-EEA1 in 15-cm dishes, with one dish per replicate. Creation of the 293^{EL} cells is described in protocol dx.doi.org/10.17504/protocols.io.byi7puhn.

2 At 80% confluency, harvest cells on ice by scraping in 2 mL of cold DPBS. Pellet at 1,000xg for 2 min at 4 ºC.

3 Discard supernatants, wash pellets once with 1 mL of cold KPBS buffer (25 mM KCl, 100 mM potassium phosphate, pH 7.2), and pellet at 1,000xg for 2 min at 4 ºC.

4 Resuspend cell pellets in 1 mL of KBPS buffer supplemented with protease and phosphatase inhibitor tablets and lyse with 30 strokes with a 2 mL Dounce homogenizer on ice.

5 Centrifuge lysed cells at 1,000xg for 5 min at 4 ºC, and transfer the post-nuclear supernatants (PNS) to new tubes on ice.

5.1 If needed to remove excess nuclear components, spin the PNS from step 5 again at 1,000xg for 5 min at 4 ºC, and transfer the final PNS to new tubes on ice.

6 Determine total protein concentration by Bradford assay, and transfer 10-20 µL of each PNS to a new tube and combine with 20 µL of RIPA lysis buffer and 10 µL of 4X LDS buffer with reducing agent for later analysis by Western blot.

7 Wash α-HA magnetic beads (60 µL of bead slurry per dish) three times with 1 mL KPBS buffer with inhibtors and resuspend in KPBS with inhibitors. Add the resuspended bead slurry to each PNS, and incubate samples at 4 ºC for 50 min with gentle rotation.

8 Separate beads from the lysate with a magnetic stand, and collect the flow through. For Western blot analysis, combine 10-20 µL of each flow through with 20 µL of RIPA lysis buffer and 10 µL of 4X LDS buffer with reducing agent.

9 Using a magnetic stand, wash beads twice with 500 µL of high salt KPBS buffer (25 mM KCl, 100 mM potassium phosphate, 150 mM NaCl, pH 7.2) with protease and phosphatase inhibitors, then wash once with normal KPBS (25 mM KCl, 100 mM potassium phosphate, pH

Citation: Hankum Park, Frances V Hundley, J. Wade Harper Endosomal and lysosomal immunoprecipitation for proteomics, lipidomics, and TEM https://dx.doi.org/10.17504/protocols.io.ewov14pjyvr2/v2

7.2) with inhibitors.

10 Elute samples by addition of 120 µL 0.5% NP-40 in KBPS with inhibitors for 30 min at 4 ºC with gentle rotation. For Western blot analysis, combine 20 µL of each eluate with 6.7 µL of 4X LDS buffer with reducing agent. Immediately process remainder of eluates or snap freeze in liquid nitrogen and store at -80 ºC until processing for mass spectrometry.

Endosomal immunoprecipitation (Endo-IP) for proteomics, TEM, and lipidomics

11 Seed 293 or 293^{EL} cells in 15cm dishes with one dish per replicate.

11.1 If treating with DNM1/2 inhibitor Dyngo4a, treat 70-80% confluent dishes with either DMSO (0.4%) or Dyngo4a (20 µM final) in serum-free DMEM for 3h. After treatment, wash cells with DMEM with 10% serum and 0.4% DMSO.

12 Harvest cells at 70-80% confluency on ice by scraping in 2 mL DPBS and pelleting at 1,000xg for 2 min at 4 ºC.

13 Discard supernatants, and wash pellets once with 1 mL of KPBS buffer (25 mM KCl, 100 mM potassium phosphate, pH 7.2) and pellet at 1,000xg for 2 min at 4 ºC.

14 Resuspend cell pellets in 500 µL of KPBS supplemented with protease inhibitor cocktail and PhosSTOP tablets and lyse with 30 strokes with a 2 mL Dounce homogenizer on ice.

15 Centrifuge lysed cells at 1,000xg for 5 min at 4 ºC, and transfer the post-nuclear supernatants (PNS) to new tubes on ice.

15.1 If needed to remove excess nuclear components, spin the PNS from step 15 again at 1,000xg for 5 min at 4 ºC, and transfer the final PNS to new tubes on ice.

16 Determine total protein concentration of each lysate by Bradford assay, and transfer 10-20 µL of each PNS to a new tube and combine with 20 µL of RIPA lysis buffer and 10 µL of 4X LDS buffer with reducing agent for later analysis by Western blot (see protocol dx.doi.org/10.17504/protocols.io.byi8puhw).

17 Wash α-FLAG M2 magnetic beads (60 µL of bead slurry per dish) three times with 1 mL KPBS buffer with inhibitors, and resuspend in the same buffer. Add resuspended bead slurry to each

Citation: Hankum Park, Frances V Hundley, J. Wade Harper Endosomal and lysosomal immunoprecipitation for proteomics, lipidomics, and TEM https://dx.doi.org/10.17504/protocols.io.ewov14pjyvr2/v2

PNS, and incubate at 4 ºC for 50 min with gentle rotation.

18 Separate beads from the lysate with a magnetic stand, and collect the flow through.

18.1 For Western blot analysis, combine 10-20 µL of each flow through with 20 µL of RIPA lysis buffer and 10 µL of 4X LDS buffer with reducing agent.

19 Using a magnetic stand, wash beads three times with 500 µL of KPBS buffer (25 mM KCl, 100 mM potassium phosphate, pH 7.2) with inhibitors.

19.1 The washed beads can be stored at -80 °C until being processed for lipidomics study.

20 Elution:

20.1 For analysis by negative stain transmission electron microscopy (TEM), elute samples by addition of 50 µL 3xFLAG peptide solution (500 µg/mL in KPBS) at 25 ºC for 45 min with gentle shaking. Transfer eluates to new tubes, and proceed to TEM analysis.

20.2 Alternatively, for organelle proteomics analysis by mass spectrometry, elute samples by addition of 120 µL 0.5% NP-40 in KBPS with inhibitors for 30 min at 4 ºC with gentle rotation. For Western blot analysis, combine 20 µL of each eluate with 6.7 µL of 4X LDS buffer with reducing agent. Immediately process the remainder of the eluates or snap freeze in liquid nitrogen and store at -80 ºC until processing for LC-MS.

Endosomal and lysosomal enrichment for APP/Aβ TOMAHAQ proteomics

21 For each replicate, seed 293^{EL}-APP* cells in 5x15cm dishes (2x15cm for Lyso-IP and 3x15cm for Endo-IP), and seed 293^{EL}-APP-/- cells in 5x15 cm dishes so that they will be approximately 60% confluent the next day and approximately 80-90% confluent two days later.

21.1 Generally, three replicates of each 293EL-APP* treatment group (e.g. DMSO or secretase inhibitors) and two replicates of 293EL-APP-/- should be processed simultaneously.

Citation: Hankum Park, Frances V Hundley, J. Wade Harper Endosomal and lysosomal immunoprecipitation for proteomics, lipidomics, and TEM https://dx.doi.org/10.17504/protocols.io.ewov14pjyvr2/v2

22 One day after seeding, treat cells with vehicle control (DMSO), GSI, GSM, or BSI to a final concentration of 2 µM and 0.2% DMSO. Incubate cells with the compounds for 15h.

23 The next day, harvest cells by discarding media and scraping in 2 mL KPBS buffer supplemented with DMSO, GSI, GSM, or BSI (note that the appropriate compound should be used in KPBS buffer throughout subsequent steps to continue inhibiting the desired enzyme).

24 Pellet cells at 1,000xg for 2 min at 4 ºC, discard supernatants, resuspend pellets in 5 mL KPBS, and pellet cells at 1,000xg for 2 min at 4 ºC.

25 Resuspend pellets in 5 mL of KPBS with the addition of protease and phosphatase inhibitors and lyse with 20 strokes with a 7-mL Dounce homogenizer and tight pestle.

26 Clarify lysate by centrifugation at 1,000xg for 5 min at 4 ºC. The lysate may be further clarified by transferring the PNS from the first spin to a new tube on ice, spinning again, and transferring the final PNS to a new tube.

27 Determine the protein concentration of each lysate by Bradford assay, and transfer 10-20 µL of each PNS to a new tube and combine with 20 µL of RIPA lysis buffer and 10 µL of 4x LDS buffer with reducing agent for later analysis by Western blot.

28 Combine 110 µL of each PNS with 183 µL of 8M urea/50mM NaCl/0.8% NP-40 buffer and store at -80 ºC for later analysis by mass spectrometry.

29 Prepare α-FLAG and α-HA magnetic beads (50 µL of bead slurry per dish) on a magnetic stand by washing three times with KPBS and resuspending in KPBS (25 µL per dish for α-FLAG beads and 50 µL per dish for α-HA beads). Add 150 µL of α-FLAG M2 beads per PNS (which came from 3x15cm dishes) and add 100 µL of α-HA beads per PNS (which came from 2x15cm dishes). Incubate samples for 45 min at 4 ºC with gentle rotation.

30 Separate beads from the flow through with a magnetic stand, and collect the flow through. For Western blot analysis, combine 10-20 µL of each flow through with 20 µL of RIPA lysis buffer and 10 µL of 4x LDS buffer with reducing agent.

31 Wash beads:

Citation: Hankum Park, Frances V Hundley, J. Wade Harper Endosomal and lysosomal immunoprecipitation for proteomics, lipidomics, and TEM https://dx.doi.org/10.17504/protocols.io.ewov14pjyvr2/v2

31.1 Wash α-FLAG beads twice with 500 µL KPBS containing the compound, and once with 1 mL KPBS without compounds.

31.2 Wash α-HA beads twice with 500 µL high-salt KPBS (KPBS with 155 mM NaCl) containing the compound, and once with 1 ml regular salt KPBS without compounds.

32 Elute samples with 5M urea/0.5% NP-40 KPBS buffer (180 µL for α-FLAG beads and 120 µL for α-HA beads) for 50 min at 30 ºC with shaking.

33 For Western blot analysis, combine 10 µL of each eluate with 3.3 µL of 4X LDS buffer with reducing agent.

34 Split the remainder of each eluate in two for future “Lyso” or “Endo” (20% of eluate) and “Lyso_LMW” or “Endo_LMW” (80% of eluate) samples, the latter of which are filtered as follows.

34.1 To detect low abundance Aβ peptides, filter samples with Amicon Ultra 0.5 mL 50 kDa centrifugal filters. Load 250 µL of each PNS onto a 50 kDa Amicon column, and reserve the remainder of the PNS to serve as the regular PNS sample.

34.2 Dilute Lyso_LMW samples with 112 µL of 5M urea/0.5% NP-40 buffer and load onto 50 kDa columns. Dilute Endo_LMW samples with 64 µL of 5 M urea/0.5% NP-40 buffer and load onto 50 kDa columns.

34.3 Centrifuge columns at 14,000 g at 10 ºC for 12 min or until residual column volume is approximately 50 µL. To increase the yield of filtered Aβ peptides, dilute residual retentate with 150 µL of 5 M urea/0.5% NP-40 buffer, and centrifuge the columns at 14,000xg at 10ºC for 12 min.

34.4 Measure the final filtrate volume and transfer to new Protein LoBind tubes. Dilute remaining, unfiltered PNS, Lyso, and Endo samples with 20 µL 5 M urea/0.5% NP-40.

35 Immediately process the remainder of the eluates or snap freeze in liquid nitrogen and store at -80 ºC until processing for proteomics study.

Citation: Hankum Park, Frances V Hundley, J. Wade Harper Endosomal and lysosomal immunoprecipitation for proteomics, lipidomics, and TEM https://dx.doi.org/10.17504/protocols.io.ewov14pjyvr2/v2