Oct 03, 2021 Version 1

Endosomal and lysosomal immunoprecipitation for proteomics, lipidomics, and TEM V.1

Nature Communications

DOI
dx.doi.org/10.17504/protocols.io.byi9puh6

Hankum Park[1,2], 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 20815, USA

Frances V Hundley
Harvard Medical School

DOI: https://dx.doi.org/10.17504/protocols.io.byi9puh6

External link: https://doi.org/10.1038/s41467-022-33881-x

Protocol Citation: Hankum Park, Frances V Hundley, J. Wade Harper 2021. Endosomal and lysosomal immunoprecipitation for proteomics, lipidomics, and TEM. **protocols.io https://dx.doi.org/10.17504/protocols.io.byi9puh6**

Manuscript citation:
Park, H., Hundley, F.V., Yu, Q. *et al.* Spatial snapshots of amyloid precursor protein intramembrane processing via early endosome proteomics. *Nat Commun* **13**, 6112 (2022). https://doi.org/10.1038/s41467-022-33881-x

Protocol status: Working

Created: September 27, 2021

Last Modified: May 31, 2024

Protocol Integer ID: 53569

Keywords: ASAPCRN, lysosomal immunoprecipitation for proteomic, lysosome purification, isolation of lysosome, electron microscopy imaging of endosome, early aspects of the endolysosomal system, sorting endosome, endosome, endolysosomal system, lysosome, lysosomal immunoprecipitation, proteomic, mitochondria, peroxisome, approach for purification, endo, lipidomic, purification, method as endo

Funders Acknowledgements:
NIH
Grant ID: NS083524
NIH
Grant ID: NS110395
Aligning Science Across Parkinson's
Grant ID: ASAP-000282

Disclaimer

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

The protocol content here is for informational purposes only and does not constitute legal, medical, clinical, or safety advice, or otherwise; content added to **protocols.io** is not peer reviewed and may not have undergone a formal approval of any kind. Information presented in this protocol should not substitute for independent professional judgment, advice, diagnosis, or treatment. Any action you take or refrain from taking using or relying upon the information presented here is strictly at your own risk. You agree that neither the Company nor any of the authors, contributors, administrators, or anyone else associated with **protocols.io**, can be held responsible for your use of the information contained in or linked to this protocol or any of our Sites/Apps and Services.

Abstract

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 method as Endo-IP. This allows us to examine the proteome, lipidome, as well as electron microscopy imaging of endosomes.

Materials

	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

	A	B	C
	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

	A	B	C
	Pierce anti-HA magnetic beads	Thermo Fisher Scientific	88837
	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

	A	B	C
	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
	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

	A	B	C
	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	

Troubleshooting

Lysosomal immunoprecipitation (Lyso-IP) for organelle proteomics

1 Seed 293 cells or 293^{EL} cells expressing TMEM192-HA and FLAG-EEA1 in 15-cm dishes, with one dish per replicate. Creation of the 293EL 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 DPBS and 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.

6 Determine total protein concentration by Bradford assay, and transfer 10 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 and resuspend in the KPBS. 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 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 cocktail, then wash once with KPBS with the 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.

16 Determine total protein concentration of each lysate by Bradford assay, and transfer 10 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 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 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 twice with 500 µL of KPBS buffer (25 mM KCl, 100 mM potassium phosphate, pH 7.2) with protease and phosphatase inhibitors cocktail, then wash once with KPBS 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 FLAG peptide solution (500 µg/mL in KPBS) at 25 ºC for 45 min with gentle shaking. Transfer eluates to new tubes, and proceed for 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 5×15cm dishes (2×15cm for Lyso-IP and 3×15cm for Endo-IP), and seed 293^{EL}-APP-/- cells in 5×15 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.

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 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 resuspend 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 3×15cm dishes) and add 100 µL of α-HA beads per PNS (which came from 2×15cm 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 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:

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 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 diluted 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 stored at -80 ºC until processing for proteomics study.