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A proximity proteomics pipeline for subcellular proteome and protein interaction mapping

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We use this protocol and it's working

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Abstract

Proximity labeling (PL) coupled with mass spectrometry has emerged as a powerful technique to map proximal protein interactions in living cells. Large-scale sample processing for proximity proteomics necessitates a high-throughput workflow to reduce hands-on time and increase quantitative reproducibility. To address this issue, we developed a scalable and automated PL pipeline, including generation and characterization of monoclonal cell lines, automated enrichment of biotinylated proteins in a 96-well format, and optimization of the quantitative mass spectrometry (MS) acquisition method. Combined with data-independent acquisition (DIA) MS, our pipeline outperforms manual enrichment and data-dependent acquisition (DDA) MS regarding reproducibility of protein identification and quantification. We apply the pipeline to map subcellular proteomes for endosomes, late endosomes/lysosomes, the Golgi apparatus, and the plasma membrane. Moreover, using serotonin receptor (5HT2A) as a model, we investigated agonist-induced dynamics in protein-protein interactions. Importantly, the approach presented here is universally applicable for PL proteomics using all biotinylation-based PL enzymes, increasing both throughput and reproducibility of standard protocols.

Materials

Reagents and Tools

	Reagent/resource	Reference or source	Identifier or catalog number
	Cell lines		
	HEK293T/17 cells	ATCC	CRL-11268
	Antibodies		
	Mouse anti-FLAG (M1)	Sigma	F-3040
	Mouse anti-Vimentin	Invitrogen	MA3-745
	Rabbit anti-E Cadherin	CST	3195T
	Mouse anti-RAB5A	Fisher	89333555
	Mouse anti-RAB9	Invitrogen	MA3-067
	Rabbit anti Golgin-97	CST	13192
	Chicken anti-GFP	VWR	RL600-901-215
	AF488-labeled goat anti-Chicken	Invitrogen	A11039
	AF555-labeled goat anti-Mouse	Invitrogen	A21422
	AF647-labeled goat anti-Rabbit	Invitrogen	A21244
	Rabbit HRP anti-GAPDH	Bio-legend	607904
	HRP anti-Streptavidin	VWR	N100
	Recombinant DNA		
	NES-APEX2	This study	N/A
	2xFYVE-APEX2	This study	N/A
	GalT-APEX2	This study	N/A
	LAMP1-APEX2	This study	N/A
	LAMTOR1-APEX2	This study	N/A
	Lyn11-APEX2	This study	N/A
	5HT2A-APEX2	This study	N/A
	Gαq-RLuc8	Addgene	140982

	Reagent/resource	Reference or source	Identifier or catalog number
	Gβ3	Addgene	140988
	GFP2-Gy9	Addgene	140991
	5HT2A	(Kim et al, 2020)	N/A
	Chemical, enzymes and other reagents		
	Dulbecco's Modification of Eagle's Medium (DMEM)	Corning	10-013-CV
	Fetal bovine serum (FBS)	Gibco	A31605-01
	Dulbecco's Phosphate-Buffered Saline (DPBS)	Corning	21-031-CV
	0.05% Trypsin-EDTA	Gibco	25300-054
	Penicillin streptomycin solution	Corning	30-002-CI
	PolyJet	SignaGen	SL100688
	0.45 μm PVDF filter unit	Millipore	SE1M003M00
	Cellstripper	Corning	25-056-CI
	Attune performance tracking beads	Thermo Fisher	4449754
	ECL western blotting substrate	Pierce	32106
	Streptavidin magnetic beads	Pierce	88817
	Biotin phenol (Biotin Tyramide)	Iris Biotech	LS-3500
	Hydrogen peroxide	Sigma	H1009-100ML
	Sodium azide	Sigma	S2002
	Sodium ascorbate	Spectrum	S1349
	Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid)	Sigma	238813
	16% Formaldehyde (w/v)	Pierce	28908
	EverBrite mounting medium with DAPI	Biotium	23002



	Reagent/resource	Reference or source	Identifier or catalog number
	cOmplete protease inhibitor cocktail tablets mini, EDTA-free	Roche	11846170001
	660nm protein assay kit	Pierce	22662
	TCEP	Pierce	20490
	Dithiothreitol (DTT)	Sigma	D0632
	Iodoacetamide (IAA)	Sigma	I1149
	Sequencing-grade modified trypsin	Promega	V5111
	Lysyl endopeptidase	VWR	100369-822
	BioPureSPE 96-well C18 plate	NEST	HNS S18V
	Ascorbic Acid	Sigma	A5960
	BSA, Free Fatty Acid	Akron	AK8909
	Coelenterazine 400a	Nanolight	340-1
	Dialyzed FBS	Omega Scientific	FB-03
	Hank's Balanced Salt Solution	Gibco	14065056
	Sodium chloride	Sigma	S7653-250G
	Tris HCl pH 7.5	Corning	46-030-CM
	Tris HCl pH 8.0	Corning	46-031-CM
	Sodium deoxycholate	Sigma	D6750-500G
	Triton-x100	Sigma	T9284-100ML
	Sodium dodecyl sulfate	Fisher BioReagents	BP166-500
	Urea	Promega	V3171
	Potassium chloride	Sigma	P9541-1KG
	Sodium carbonate	Sigma	S2127-500G
	Pierce 660 nm Protein Assay Reagent	Thermo Scientific	22660
	Software		



	Reagent/resource	Reference or source	Identifier or catalog number
	Spectronaut (version 16.0)	Biognosys	https://biognosys.com/shop/spectronaut
	MSstats (version 4.4.0)	Bioconductor	https://www.bioconductor.org/packages/release/bioc/html/MSstats.html
	Attune NxT software	Thermo Fisher Scientific	
	FlowJo	FlowJo, LLC	https://www.flowjo.com/
	Fiji	imageJ	https://imagej.net/software/fiji/
	NIS-Elements software (v. 5.30.01 build 1541)	Nikon	
	Prism (v8.0)	GraphPad Software	
	Other		
	Orbitrap Exploris 480 MS with internal calibration option	Thermo Fisher Scientific	BRE725533
	EASY-nLC 1200 system	Thermo Fisher Scientific	
	Nikon Ti2-E microscope	Nikon	
	Attune NxT Flow Cytometer	Thermo Fisher Scientific	
	KingFisher Flex system	Thermo Fisher Scientific	



Cell culture

- 1 Seeding 3-4 million cells in a 10-cm dish or 500K cells in a 6-well plate on Day One. The cells will be ~70% confluent after 48 hours.
- 2 Add doxycycline (final concentration of 1ug/mL) to induce cells for 24 hours on Day Two.

APEX proximity labeling

- 3 Perform APEX proximity labeling on Day Three.
- 4 Incubate cells with 500uM BP in complete medium pre-warmed to 37 °C for 30min.
- 5 Add 2mM H₂O₂-containing medium (DMEM+10% FBS) to the 10-cm dish or 6-well plate to have a final concentration of 1mM H₂O₂.
- 6 Allow the reaction to go for 45sec at room temperature.
- 7 Invert the dish/plate and pour out the medium.
- 8 Immediately wash the cells 3x with ice-cold quencher solution (PBS+ 5mM Trolox, 10 mM sodium azide, 10 mM sodium ascorbate) for 1min (8mL for 10cm dish or 1mL for 6-well plate). Aspirate or invert to remove.
- 9 Collect the cells in fresh quencher solution (8ml for 10cm plate or 1mL for 6-well plate) and pellet the cells by centrifugation at 3000g for 10min at 4 °C, remove supernatant and continue to cell lysis or freeze cell pellets in dry-ice for storage

Cell lysis

- 10 Lyse cells in 1ml RIPA buffer supplement with protease inhibitors, antioxidants, and DTT
- 11 Perform a freeze-thaw cycle on dry ice and thaw in a  37 °C water bath



- 12 Sonicate samples (5 seconds, 15% Duty Cycle x 2)
- 13 Transfer samples to 1.5ml Eppendorf tubes
- 14 Centrifuge samples at 13,000xg for 10min at  4 °C and save the supernatant.
- 15 Take a small amount (25 uL) of the lysate for WB analysis
- 16 Quantify protein concentration (supernatant) using 600nM Pierce Assay Kit with Detergent Compatibility Reagent (optional)

Automated enrichment protocol for biotinylated proteins in Kingfisher

- 17 For the automated biotinylation enrichment protocol, the Kingfisher Flex system (Thermo Fisher) is programmed to simultaneously process a maximum of 96 samples. This protocol below includes two parts, where part 1 (Plate 1-3) is for washing magnetic streptavidin beads and binding of the biotinylated proteins to beads, and part 2 (Plate 4-12) is for washing and collecting beads prior to Lys-C/trypsin digestion. The enrichment protocol is conducted in the cold room using deep-well plates.
- 18 **1st day, protocol: Biotinylation APEX-MS part 1**
 - 18.1 **Plate 1 (1 mL, stock)**
 - a. RIPA buffer plate 1
 - b. Add 80 uL of streptavidin beads to each well of 1 mL of RIPA buffer
 - 18.2 **Plate 2 (1 mL, stock)**
 - a. RIPA buffer plate 2
 - b. 1 mL of RIPA buffer
 - 18.3 **Plate 3 (1 mL, fresh lysis)**
 - a. Sample binding plate
 - b. All samples from lysis are added to the plate
 - c. Beads are transferred to this plate after 2x RIPA wash and left in this plate for overnight binding
 - d. Tip comb is left in this plate at the end of the protocol



19 **2nd day, protocol: Biotinylation APEX-MS part 2**

- 19.1 Plate 4-6 (1 mL, stock)
 - a. RIPA buffer plates 4-6
 - b. Continued from Plate 3
 - c. Beads with bond samples are collected from Plate 3 and transferred to these plates for 3x RIPA wash
- 19.2 Plate 7 (1 mL, stock)
 - a. KCl solution wash plate (1M KCl)
 - b. Stock solution prepared and can be stored for a long time
- 19.3 Plate 8 (1 mL, stock)
 - a. Na₂CO₃ solution wash plate (0.1M Na₂CO₃)
 - b. Stock solution prepared and can be stored for a long time
- 19.4 Plate 9 (1 mL, fresh)
 - a. 2M urea in 50mM Tris-HCl [pH=8.0] wash plate
 - b. 1 M Tris-HCl is ready
 - c. Urea prepared freshly each time
- 19.5 Plate 10-11 (1 mL, fresh)
 - a. 50mM Tris-HCl [pH=8.0] wash plates 10-11
 - b. Prepared from 1M Tris-HCl solution
- 19.6 Plate 12 (200 uL, fresh)
 - a. Digestion plate with 2M urea in 50mM Tris-HCl [pH=8.0] buffer
 - b. Beads and tip comb are left in this plate

Protein digestion

- 20 Reduce proteins by adding 5mM TCEP (final concentration, add 2ul of a 500mM TCEP solution) and incubate by shaking at 1000rpm @37 degrees for 30min.
- 21 Protein alkylation by adding iodoacetamide to 5mM (final concentration, add 2ul of a 500mM iodoacetamide solution) and incubate by shaking at 1000rpm @RT for 30min.
- 22 Quench IAA with 5mM DTT (final concentration, add 2ul of a 500mM iodoacetamide solution).
- 23 Add 2ul of trypsin (~1ug), and 1 ul of Lys-C (2ug/ul) and incubate O/N at 37 degrees under shaking at 1000 rpm. The on-bead digestion is set to 37 °C for 6h and RT for 12h.



24 Following morning add additional 0.5 ug of trypsin and incubate an additional 2h at  37 °C .

25 Transfer supernatant to a new 96-well plate using magnetic rack, and acidify sample with 10% trifluoroacetic acid (TFA) to ~pH2 (~0.5% TFA final).

C18 desalting

26 The peptide samples are desalted using C18 96-well plate (BioPureSPE, HNS S18V-20mg, the Nest group)

27 Wash the plate three times with 100 µL 80% acetonitrile (ACN)/0.1% TFA by centrifugation at 800 rpm for 1 min

28 Equilibrate the plate three times with 100 µL 2% ACN/0.1% TFA by centrifugation at 1200 rpm for 2 min

29 Load samples by centrifugation at 1600 rpm for 2 min

30 Re-load samples by centrifugation at 1600 rpm for 2 min

31 Wash the plate 3 times with 100 µL 2% ACN/0.1% TFA by centrifugation at 1600 rpm for 2 min

32 Elute twice with 55 µL 50% ACN/0.25% formic acid (FA) by centrifugation at 1600 rpm for 2 min

33 Dry the samples by vacuum centrifugation (~2h).

34 Store the dried samples at  -20 °C or resuspend them in 20 uL 0.1% formic acid for mass spec analysis.