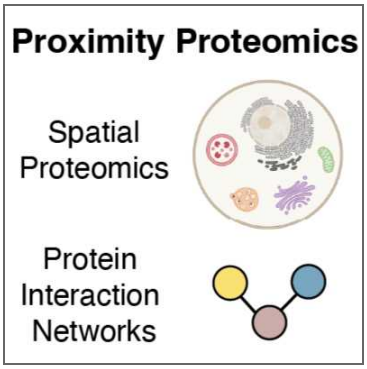

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We use this protocol and it's working

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A proximity proteomics pipeline for subcellular proteome and protein interaction mapping

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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

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	Reagent/resource	Reference or source	Identifier or catalog number
	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
	Gβ3	Addgene	140988
	GFP2-Gγ9	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

	Reagent/resource	Reference or source	Identifier or catalog number
	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
	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

	Reagent/resource	Reference or source	Identifier or catalog number
	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		
	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	

	Reagent/resource	Reference or source	Identifier or catalog number
	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 ^{O}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 ^{o}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 K...

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 ^{O}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.