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APEX2-based proximity biotinylation of NLRP3 and P4C (SidC) during inflammasome activation

Forked from Proximity biotinylation of ATG8 proteins and selective autophagy receptors

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ABSTRACT

APEX2-based proximity labeling for the discovery of proteins proximal to NLRP3. Aspects of this APEX2 protocol can be applied to other target proteins.

PROTOCOL REFERENCES

https://pubmed.ncbi.nlm.nih.gov/25419960/
https://pubmed.ncbi.nlm.nih.gov/26866790/
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10620096/

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We use this protocol and it's working

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MATERIALS

Cell culture

PBS; Phosphate buffered saline: ThermoFisher (#14040133)
Dulbecco's MEM (DMEM), high glucose, pyruvate (Gibco / Invitrogen, 11995)
Penicillin-Streptomycin (10,000 U/mL) (Thermo 15140163)
GlutaMAX™ Supplement (Thermo 35050061)

Chemicals, Peptides, and Recombinant Proteins

Protease Inhibitor Cocktail **Roche Catalog #**11873580001
PBS (10x) **Santa Cruz Catalog #**sc-24947
tris(2-carboxyethyl)phosphine (TCEP) **Gold Biotechnology Catalog #**TCEP2
Formic Acid (FA) **Sigma – Aldrich Catalog #**94318
Acetonitrile (ACN) **Sigma – Aldrich Catalog #**34851
Trifluoroacetic acid (TFA) (fisher scientific, A11650)
HPLC Water (Sigma 270733)
Sodium Chloride **Sigma – Aldrich Catalog #**S9888
MOPS **Sigma – Aldrich Catalog #**M1254
Sequencing grade Trypsin **Promega Catalog #**V5111
Lys-C **Wako Chemicals Catalog #**129-02541
EPPS **Sigma – Aldrich Catalog #**E9502
2-Chloroacetamide **Sigma – Aldrich Catalog #**C0267
DTT (GoldBio, DTT10)
Biotin phenol (LS-3500.0250, Iris Biotech)
Potassium chloride, KCl (Sigma #P9541)
Sodium chloride, NaCl (Sigma 105012)
Trolox (Cayman chemicals, 10011659)
Sodium azide (Sigma S2002)
(+)-Sodium L-ascorbate (Sigma A4034)
Sodium carbonate (7527-04)
Urea (Sigma U5378)
Nigericin (N7143)
CL097 (Sigma SML2566)
LPS (Invivogen, tlrl-b5lps)
NP-40 alternative (PRI, N59000)
SDS, Sodium Dodecyl Sulfate (Bio-Rad #1610302)
BSA, 200 mg/mL standard (Sigma, P5369)

Buffers and solutions

Lysis buffer: 50 mM Tris HCl pH 7.5, 150 mM NaCl, 1.0% (v/v) SDS, 1.0 % (v/v) NP-40, 1X cOmplete™ Protease Inhibitor Cocktail

EPPS buffer, 200 mM EPPS, pH 8.5 (Fisher scientific AAJ61476AE)

30% (wt/wt) H_2O_2 reagent (Sigma H1009; NOTE: Do not keep more than ~6 months)

Critical Commercial Assays

Tandem Mass Tags **Thermo Fisher Scientific Catalog #**90406

DC protein assay II **Bio-Rad Laboratories Catalog ##**5000112

Mass Spec

Empore™ SPE Disks C18 **Catalog #**2215

Pierce™ High pH Reversed-Phase Peptide Fractionation Kit **Thermo Fisher Catalog #**84868

- Orbitrap Fusion Lumos Mass Spectrometer, ThermoFisher Scientific, Cat#IQLAAEGAAPFADBMBHQCat#IQLAAEGAAPFADBMBHQ [or equivalent]

Other

Streptavidin-coated agarose beads: Pierce (#88817)

Cell line construction and validation (brief overview)

1 The choice of cells, constructs, proximity biotinylation enzyme (e.g., TurboID vs. APEX2), treatments, etc. to employ for proximity biotinylation experiments depends on the experimental design. Here, mouse immortalized bone-marrow derived macrophages iBMDM cells (Gift from Kate Fitzgerald), either wild-type or Nlrp3-knockout, were reconstituted with Nlrp3-APEX2-FLAG-IRES-GFP (Addgene #218633) or FLAG-APEX2-P4C-IRES-mNeonGreen (Addgene #218646). Adherent cells are important for the washing steps of this protocol, but it can be adapted for suspension cells with the addition of spin steps during quenching.

1.1 These cell lines were grown in Dulbecco's modified Eagle's medium (DMEM, high glucose and pyruvate) supplemented with 10% fetal bovine serum, GlutaMAX™, and Penicillin-Streptomycin. They were maintained in a 5% CO_2 incubator at 37 ^{o}C. iBMDMs were split 1:10 every other day, but growth rates vary among different cell types and passages.

1.2 The appropriate iBMDMs were spinfected with Nlrp3-APEX2-FLAG-IRES-GFP (Addgene #218633) or FLAG-APEX2-P4C-IRES-mNeonGreen (Addgene #218646). 3 - 5 d after spinfection, cells were bulk sorted for low GFP+/mNeonGreen+ expressors with a Sony SH800 Cell Sorter operating in purity mode.

Note

An extended protocol for producing virus is available here: https://www.addgene.org/protocols/lentivirus-production/?utm_term=&utm_campaign=Primary+Ad+Group:+Website,+blog,+collections.&utm_source=adwords&utm_medium=ppc&hsa_acc=3245806047&hsa_cam=112133441&hsa_grp=63724608643&hsa_ad=320492093642&hsa_src=g&hsa_tgt=dsa-596073738403&hsa_kw=&hsa_mt=&hsa_net=adwords&hsa_ver=3&gad_source=1&gclid=Cj0KCQjwztOwBhD7ARIsAPDKnkBDRe1ZLOhpLmjku0S2lwpO2RobkQ5ylaJPMUZ4Sofxe5MhJOULGNAaAvVrEALw_wcB

2 Check protein expression levels by comparing LPS-primed WT iBMDMs to LPS-primed Nlrp3-APEX2-FLAG iBMDMs using Western blot.

3 Check protein localization by IF using an anti-FLAG antibody.

Note

An extended protocol for immunofluorescence is available here: dx.doi.org/10.17504/protocols.io.kxygxyeeol8j/v1

4 Validate functional reconstitution of Nlrp3-APEX2 in Nlrp3-KO iBMDMs with LDH release.

Note

An extended protocol for LDH and IL-1β release is available here: dx.doi.org/10.17504/protocols.io.kxygxyeeol8j/v1

APEX2 proximity biotinylation

5 Prepare the following ahead of time: Biotin-phenol/biotin tyramide (500 mM in DMSO, aliquot, store at -80 °C)

6 Grow cells to just fully confluent in 10-cm dishes. For certain iBMDMs, for example, a 1:10 split produces a fully confluent dish 2 d later. Cells should be seeded in 10 mL complete DMEM.

7 For the cell harvesting day, freshly prepare: 100X Trolox in DMSO (500 mM, requires sonication), 100X Sodium ascorbate in PBS (1 M), 100X sodium azide in PBS (1 M). Each of these solutions is 1:100X, so prepare enough for the amount of quenching buffer used (35 mL buffer per replicate).

8 Stagger treatments so that only 3-4 plates (for example, 1 group of replicates) are harvested at a time. For inflammasome treatments, this could mean staggering LPS treatments for different conditions by 1 h. Regardless, replace the medium with 10 mL fresh DMEM/etc.

Note

When treating cells with LPS for inflammasome experiments, exchange the cell culture medium into 10 mL fresh DMEM lacking serum. Serum starvation, plus some degree of contact inhibition from a fully confluent plate, can reduce the amount of mitotic cells and consequently ensure more cells can undergo an NLRP3 response.

All plates should receive fresh medium with the same components, for similar amounts of time, if a media swap occurs. For example, if you want to compare +/- LPS, also swap the -LPS plate into fresh media for 4 h prior to harvesting.

8.1 Add 10 µL biotin phenol (BP, 500 mM) to cells 45 mins prior to collecting them. For example, for 4 h LPS priming only, add BP to plates 3 h 15 min after LPS. For a 30 min nigericin treatment, add BP to plates 3 h 45 min after LPS treatment, nigericin at 4 h LPS, and collect cells 30 min later.

Note

BP is not very soluble in cell culture medium and will likely crash out before dissolving into solution, so add it to the cells while swirling the plate and continue swirling until all it goes into solution.

9 Just before harvesting the cells, prepare the following:

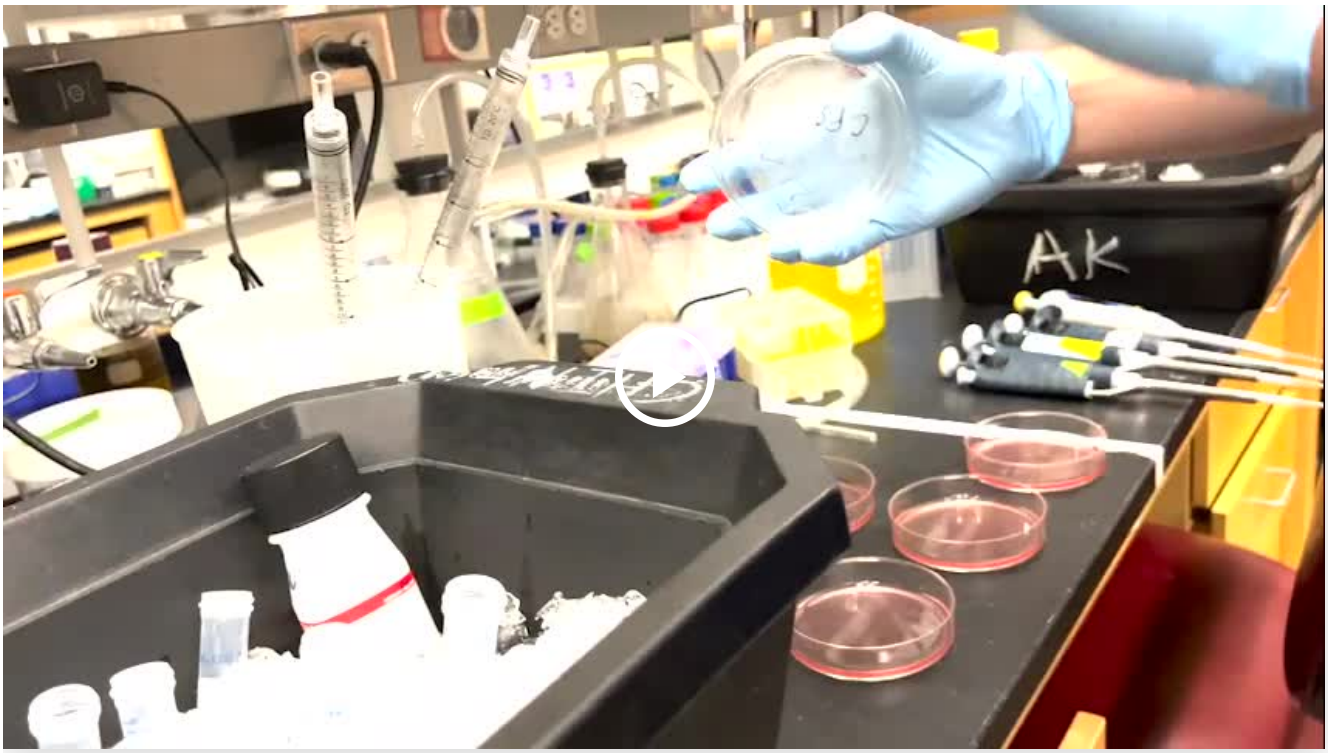
9.1 100 mM H_2O_2 in DPBS (10 uL of 30% H_2O_2 into 990 µL DPBS). Prepare individual pipettes with 100 uL of H_2O_2 for each plate being harvested at a time.

9.2 3 falcon tubes per plate, with 10 mL ice-cold quenching buffer in each.

> **Note**
>
> Make up fresh batches of quenching buffer by diluting 100x Trolox, 100x Sodium ascorbate, and 100X sodium azide into ice-cold PBS with vigorous stirring (to 1X). Each replicate will need 35 mL quencher buffer.

9.3 Trays of ice for harvesting cells.

10 Bring cells to the bench and harvest them (see video and text below).



Note

In the video, we include an additional PBS wash to remove quencher--this isn't necessary, because the low carryover of quencher will not affect protein quantification with the DC protein assay II kit.

10.1 Quickly pipette H_2O_2 into each of the plates, swirl, and incubate at RT for 1 min.

10.2 Pour off the solutions and quickly add ice-cold quencher solution to each plate, and place them on ice.

10.3 Aspirate the quencher and wash the plates two additional times. Scrape the cells in the final 10 mL quencher solution, transfer to a falcon tube, and scrape the plates with an additional 5 mL quencher solution to harvest residual cells.

10.4 Centrifuge cells (1000 xg, 5 min), aspirate the quencher solution, and flash-freeze pellets in liquid nitrogen. Store at -80 ^{O}C until ready for immunoprecipitation.

10.5 Repeat with additional replicates at staggered timepoints to prevent an overwhelming amount of simultaneous bench work.

Biotin-streptavidin immunoprecipitation and on-bead digestion

11 Prepare lysis buffer: 50 mM Tris HCl pH 7.5, 150 mM NaCl, 1.0% (v/v) SDS, 1.0 % (v/v) NP-40, 1X cOmplete ™ Protease Inhibitor Cocktail

Note

Lysis buffer can be kept slightly chilled but cannot be put on ice, because the 1% SDS will precipitate. Work SDS powder in a hood, if applicable. Detergents and other components can be dissolved overnight, but the protease inhibitor cocktail should be added immediately before proceeding.

12 Rapidly thaw cell pellets by submerging tubes in warm water for ~30 s, then place them on ice.

13 Gently vortex the stock of magnetic streptavidin beads for ~30 s to thoroughly resuspend them. Aliquot 50 µL beads (slurry) per replicate, and ~10% excess for pipetting loss, into a protein LoBind microcentrifuge tube using a wide bore P1000 pipette tip. Wash 3x with lysis buffer, and then rotate end-over-end while preparing other reagents.

14 Add 1 mL of lysis buffer to each cell pellet and immediately transfer them to microcentrifuge tubes at RT.

Note

Upon addition of lysis buffers, pellets will quickly becoming viscous because the loss of nuclear integrity releases DNA. These solutions will be very hard to pipette, so immediately pipette off the pellet into a LoBind tube before pipetting up and down to resuspend the pellet.

15 Sonicate each lysate for ~10 seconds with a small probe sonicator.

15.1 Repeat this step once.

16 Add 0.25 µL benzonase to each tube. Invert tubes at RT for 30 min to complete lysis and DNA digestion. The solutions should be much less viscous following sonication and benzonase treatment.

17 Spin tubes at 15,000 xg for 10 min. There shouldn't be a large pellet.

18 Quantify each of the supernatants with the DC protein assay kit II. Use reagent A'.

18.1 For a fully confluent 10 cm plate lysed in 1 mL, dilute the clarified lysates 1:5 in lysis buffer to be in the linear range of the assay.

18.2 Run a BSA standard curve alongside the APEX2 lysates by diluting 200 mg/mL analytical standard BSA solution to 4 mg/mL in lysis buffer. Perform several 2-fold serial dilutions to make a 6-point curve (4, 2, 1, 0.5, 0.25, 0.125 mg/mL)

19 Normalize protein concentrations to 2 mg/mL. Prepare a total volume of 1075 µL in fresh protein LoBind microcentrifuge tubes. Remove 75 µL of this solution into pre-prepared microcentrifuge tubes containing 25 µL 4XLDS sample buffer ("INPUT" sample).

normalization_curves_example.xlsx

20 Add 50 µL of bead slurry to each microcentrifuge tube containing 1 mL clarified lysate at 2 mg/mL. Resuspend or invert the beads constantly to ensure they do not settle to the bottom during aliquotting. Invert the mixtures end-over-end for 2 h at RT. 2h

Note

Note: depending on the washes with lysis buffer, the equivalent volume to achieve 50 µL of slurry per condition might have changed.

21 Wash the beads thoroughly with several solutions, using a magnetic rack to pellet the beads. Resuspend the magnetic beads with a wide bore pipette when changing solutions and place them on an end-over-end rotator while resuspending all of the replicates. Ensure that the beads don't aggregate, and if they do, gently pipette up and down with a wide bore P1000 to disperse them.

21.1 Wash 1: 1 mL lysis buffer

21.2 Wash 2: 1 mL lysis buffer

21.3 Wash 3: 1 mL 1 M KCl

21.4 Wash 4: 1 mL 100 mM Sodium carbonate (Na_2CO_3)

21.5 Wash 5: 1 mL 2 M urea in 10 mM Tris-HCl (pH 8.0)

21.6 Wash 6: 1 mL PBS. Use this wash to transfer the beads to new protein low-bind tubes.

> **Note**
>
> Transferring to new tubes at some point during the washes is important because digestion will be on-bead, and therefore contaminants stuck to the walls of the microcentrifuge tubes can carry-over to the MS data.

21.7 Wash 7: 1 mL MQ/ultrapure water.

> **Note**
>
> If transferring to an MS facility for the on-bead digestion and the remainder of the sample prep, check with them prior to proceeding with the steps below.

22 Reduction: pellet the beads and resuspend them in 75 µL EPPS pH 8.5 (200 mM) with 1.5 µL TCEP (0.5 M, ~10 mM final). Vortex, and incubate tubes at RT for 30 min. 30m

23 Alkylation: add 3 µL of freshly prepared 0.5 M chloroacetamide (in water) to each tube (final: ~20 mM). Vortex and incubate for 20 min in the dark (30 minutes max, and longer is bad). 20m

24 Quench the chloroacetamide with DTT (3.5 µL, 0.5 M), vortex, and pellet on a magnetic rack.

25 Wash beads 2x with 1 mL HPLC water to remove chloroacetamide, TCEP, and DTT.

26 Resuspend beads in 75 µL EPPS supplemented with 1 µL LysC. Shake (600-800 RPM) for 2-4 h at 37 ^{o}C (ideally, with a heated lid to prevent evaporation). 4h

27 Add 1 µL trypsin per tube. Vortex briefly and shake (600-800 RPM) for 16-24 h at 37 ^{o}C (ideally, with a heated lid to prevent evaporation). 16h

Mass spec prep 2h 15m

28 Vortex beads, pulse centrifuge them, and then put them on a magnetic rack. Remove the digested peptide flow through to a new low-bind microcentrifuge tube.

29 Wash the beads with 30 µL acetonitrile (ACN), and combine the flow through into the new tube.

30 Pellet the combined flow-through on a magnetic rack and take the flow-through, avoiding additional bead carry-over.

> **Note**
>
> This additional magnetic rack step is to remove as much bead as possible.

31 Add 5 µL TMT label reagent (12.5 µg/µl in ACN) to each vial. Vortex and then shake (600-800 RPM) for 2 h a 2h RT.

32 In the last 30 min of the TMT labeling, prepare a stage tip (6 small cookies from Empore SPE C18 disks, or a commercial C18 column) and equilibrate it:

> Note
>
> Never let the stage tip completely dry until the elution step

32.1 200 µL 100% Methanol

32.2 200 µL 80% ACN

32.3 200 µL 5% ACN/1% Formic acid (FA)

33 Place 160 µL of 1% FA in a microcentrifuge tube. Pipette 5 µL (~5 % of output) of each labeled sample into tube, vortex, and then proceed immediately to stage tip. Place remaining TMT-labeled peptides at -20 ^{o}C until after a ratio check.

34 After the sample flows through the stage tip, wash it with 200 µL 5% ACN/1% FA.

34.1 Elute the stage tip into an MS vial with 80 µL of 80% ACN

35 Dry the sample in a speed vacuum and then resuspend the dried peptides in 5% ACN/5% FA

36 Perform a ratio check search with MS. Ensure that TMT labeling is >95%, otherwise add more TMT labeling reagent to each tube and repeat the stage tip + ratio check.

37 Quench each tube with hydroxylamine at a final concentration of 0.5% (wt/v). With the volumes above, ad 15m
1.1 µL of 50% (wt/v) hydroxylamine solution. Vortex then incubate at RT for 15 min.

38 Pool equal amounts of peptides from each channel depending on the ratio check search. Note potential outliers from the ratio check search (e.g., one sample that was not represented, or was very low input).

> **Note**
>
> If one channel is far below the remainder of the channels, we often load based on the second lowest channel.

39 Dry samples in the speed vacuum. This will likely take several hours (> 4 h).

40 Fractionate the sample with the High pH Reversed-Phase Peptide Fractionation Kit (Thermo 84868). Follow most of the manufacturer's instructions, but elute with the following 12 fractions:

40.1

	A	B	C	D	E
	Step elution reagents	ACN (%)	amount (µL)	**Acetonitrile**	Triethylamine (0.1%)

	A	B	C	D	E
	Wash	5	500	25	475
	Fraction 1	10	500	50	450
	Fraction 2	12.5	500	62.5	437.5
	Fraction 3	15	500	75	425
	Fraction 4	17.5	500	87.5	412.5
	Fraction 5	20	500	100	400
	Fraction 6	22.5	500	112.5	387.5
	Fraction 7	25	500	125	375
	Fraction 8	35	500	175	325
	Fraction 9	50	500	250	250
	Fraction 10	60	500	300	200
	Fraction 11	70	500	350	150
	Fraction 12	80	500	400	100

41 Combine fractions 1 and 7; 2 and 8; 3 and 9; 4 and 10; 5 and 11; 6 and 12. This concatenates the sample space to 6 fractions.

42 Dry these fractions on a speed vacuum. They will dry at different rates, and likely take between 4 and 8 h.

43 Resuspend samples in 200 µL of 3% ACN/1% FA. Check that pH is ~2, otherwise, further acidify with small amounts of 100% FA.

44 Prepare 6 stage tips (12 small cookies from Empore SPE C18 disks, or a commercial C18 column) and equilibrate them:

44.1 200 µL 100% Methanol

44.2 200 µL 80% ACN

44.3 200 µL 5% ACN/1% Formic acid (FA)

44.4 Load sample. Collect this flow-through and save it at -20.

44.5 200 µL 5% ACN/1% Formic acid (FA)

44.6 Elute stage tips into an MS vials with 80 µL of 80% ACN

45 Dry these stage-tipped fractions on a speed vacuum. They will dry at different rates, and likely take between 2 and 4 h.

46 Resuspend the dried peptides in 8 µL of 5% ACN/5% FA, and proceed to MS.