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Cross-Linking and Strong Cation Exchange (SCX) Fractionation

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We use this protocol and it's working

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Abstract

This is a method for performing Lys-Lys cross-linking on a purified organelle sample, in this case EEA1-positive early endosomes. The crosslinker used is DSSO ((Bis(2,5-dioxopyrrolidin-1-yl) 3,3′-sulfinyldipropionate)). This cross-linker has a mass spec cleavable sulfoxide, which facilitates identification of individual cross-linked peptides.

Materials

disuccinimidyl sulfoxide (DSSO; Thermo Scientific, A33545)
2-chloroacetamide (Sigma-Aldrich, C0267)
trypsin (Promega, V511C)
Lys-C (Wako Chemicals, 129-02541)
Sep-Pak C8 50mg Cartridge (Waters, WAT054965)
EPPS (3-[4-(2-Hydroxyethyl)-1-piperazine]propanesulfonic acid) (Thermo Scientific, J61296AE)
dithiothreitol (DTT)
Empore SPE Disks C18 to generate stage tips(Sigma Millipore, 66883-U)
PolyLC PolySulfoethyl A column (3 µm particle size, 2.1 mm inner diameter, and 100 mm length) (#102SE03--)
Acetonitrile
Formic acid
Lys-C (Wako Chemicals, 129-02541)

Troubleshooting

Organelle preparations

1 Samples for cross-linking (e.g. individual organelles) are isolated using dedicated protocols such as https://doi.org/10.17504/protocols.io.ewov14pjyvr2/v2 for isolation of EEA1-positive early endosomes

Protein cross-linking

2 Resuspend freshly prepared purified organelle pellets in KPBS (25 mM KCl, 100 mM potassium phosphate, 150 mM NaCl, pH 7.2) to a protein concentration of ~1µg/uL.

3 Cross-link the proteins by adding 1 mM DSSO (Bis(2,5-dioxopyrrolidin-1-yl) 3,3′-sulfinyldipropionate) and incubate at room temperature for 40 minutes.

4 Quench the cross-linking reaction by adding 50 mM Tris buffer (pH 7.5) and incubate at room temperature for 30 minutes.

4.1 Alternatively, other cross-linker reagents could be use. For DHSO/DMTMM, 8 mM DHSO (3,3'-sulfinyldi(propanehydrazide)) and 16mM DMTMM (4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride) at 37°C for 90 min.
Proceed directly with protein precipitation. Samples can be digested using S-trap sample preparation following mini-spin column digestion protocol as provided by the manufacturer (Protifi, C02-mini-80).

Protein digestion

5 Denature the cross-linked samples by adding 8 M urea.

6 Reduce the proteins by adding 5 mM dithiothreitol (DTT) and incubate for 30 minutes at 37°C.

7 Alkylate the reduced proteins with 40 mM chloroacetamide for 30 minutes at room temperature.

8 Digest the cross-linked proteins with Lys-C enzyme at a ratio of 1:75 (enzyme) and incubate at 37°C overnight.

9 Dilute the urea concentration of the sample to 2 M using 50 mM EPPS buffer (pH 8.0).

10 Further digest the proteins by adding trypsin at a ratio of 1:100 (enzyme) and incubate at 37°C for 6 hours.

11 Desalt the peptides using Sep-Pak solid-phase extraction column.

12 Dry the desalted peptides in a SpeedVac concentrator. Time will depend on the vacuum strength.

Peptide separation by SCX

13 Fractionate the dried peptides by strong cation exchange (SCX) chromatography.
A 70-minute linear gradient at flow rate 0.18 mL/min was used as follows:
- 0% to 8% of mobile phase (0.5 M NaCl in 20% ACN, 0.05% formic acid) over 14 minutes.
- Increase to 20% at 28 minutes.
- Increase to 40% at 48 minutes.
- Increase to 90% at 68 minutes.

14 Collect fractions every 30 seconds starting at 35 minutes for 10 minutes, and then every minute thereafter.

15 Dry the collected fractions in a SpeedVac concentrator.

16 Desalt the dried fractions using StageTip.

17 Reconstitute fractions in 5% acetonitrile (ACN) and 5% formic acid.

18 Analyze the reconstituted fractions by liquid chromatography-tandem mass spectrometry (LC-MS/MS).