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APEX2 labelling and screening for biotinylated proteins by proteomics

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DISCLAIMER

Protocol under optimization

ABSTRACT

Enzyme-catalyzed proximity labeling (PL) combined with mass spectrometry (MS) has emerged as a revolutionary approach to reveal the subcellular proteomes. APEX (engineered ascorbate peroxidase) catalyzes the oxidation of biotin-phenol to the short-lived biotin-phenoxyl radical in the presence of hydrogen peroxide. This radical reacts with electron-rich amino acids such as tyrosine on neighboring proteins resulting in their biotinylation. APEX2 technology uses a mutant enzyme that increases sensitivity. This protocol describes APEX2 labelling and sample procurement for mass spectrometry from SNc and striatum.

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MATERIALS TEXT

Cutting Solution

* dilute to 1x add 1x Na bicarbonate, Na ascorbate, MgCl2 and CaCl2
-make 200ml per mouse, and store in the -80°C until just frozen

Compound	final concentration (mM)		
Choline	110		
MgCl2	7		
KCl	2.5		
NaH2PO4*2H2O	1.25		
CaCl2	0.5		
Glucose	10		
Na ascorbate	1.3		
Na bicarbonate	25		

aCSF

-initial incubation in 0.5mM of BP (-20°C) and 1uM TTX
-quenching solution add 10mM Trolox, 20mM sodium ascorbate, and 10mM sodium azide

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aCSF			
Compound	final concentration (mM)		
KCl	2.5		
Glucose	10		
NaCl	125.2		
NaH2PO4*H2O	0.3		
MgCl2*6H20	1.3		
CaCl2*2H2O	2.4		
NaHCO3	26		

Hydrogen Peroxide

Prepare 1M H2O2 in PBS and dilute to a final concentration of 1mM for reaction initiation
*prepare fresh daily
i.510ul of H2O2 into 5ml of H2O 1M solution
ii.700µl of 1M solution into aCSF

Tissue Lysis Buffer

*make roughly 1ml per reaction

Lysis Buffer	final concentration mM
Tris	50
NaCl	150
EDTA	10
Triton	1%
Trolox	5
Sodium Ascorbate	10
Sodium Azide	10
protease inhibitor	1x

TCA Solution

*TCA solution is a 100% solution in glass amber bottle in 4°C. Stable for ~23weeks
-dilute TCA to working concentration of 55%, make fresh daily

Acetone

-store in -20°C 1 day before use

-I got mine from the Decaen lab but they don't have a lot so we might need to consider getting our own.

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-Need ~5ml per reaction

Urea Dissolve Buffer

***1ml per sample**

	final concentration (M)
Urea	8
SDS	1%
Sodium phosphate Buffer, pH 8	0.1
ammion bicarbonate	0.1

*Prepare
Sodium phosphate buffer by mixing 1M NaH2PO4 (monobasic) and 1 M Na2HPO4 (dibasic)
http://cshprotocols.cshlp.org/content/2006/1/pdb.rec8303

Urea Detergent Wash Buffer

***~3ml per sample Day1, and ~2ml per sample Day 2

Urea Detergent Wash Buffer	final concentration (M)	
Urea		4
SDS		0.50%
sodium phosphate buffer	0.1	

Urea Wash Buffer (NO SDS)

*~2ml per sample

Urea Wash Buffer		final concentration (M)
Urea		4
sodium phosphate buffer	0.1	

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APEX2 Labelling

1 Construct: AAV5-CAG-DIO-APEX2NES is injected into the ventral midbrain (VM) of DATIRES-Cre mice

2 Confirmation of cellular specificity: Representative slices of SN and Str stained for TH, V5 and merged

3 Brains perfused with aCSF by trascardial perfusion with 10ml ice cold cutting solution

4 Acute slice preparation as 300µm coronal slices

5 Incubation of slices with 0.5mM BP in oxygenated artificial cerebrospinal fluid (aCSF) during the slice recovery period (1hr RT)

6 Rapid biotinylation: rapid labeling with 1 mM H2O2 in aCSF for 3 minutes

7 Rapid quenching by transferring slices to quenching aCSF (20mM Sodium ascorbate, 10mM NaN3 and 10mM Trolox) for 5 minutes.

8 Slices were transferred to ice-cold quenching aCSF for rapid dissection, tissue were flash frozen and stored in -80C for downstream western blot or proteomics. Tissue lysis and capture biotinylated proteins for proteomics

Tissue lysis and capture of biotinylated proteins for proteomics

9 Frozen tissues or synaptosome pellets were homogenized on ice in a glass dounce homogenizer with 30 strokes (in 750ul lysis buffer with protease and phosphatase inhibitor). One mouse/sample for striatum and 2mice/sample for SNc.

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10 Lysates with addition 39ul of 10% SDS were rotated for 15 min at 4°C

11 Lysates were clarified by centrifugation at 21,000 × g for 10 min at 4°C

12 Supernatants were transferred to a new prechilled Eppendorf tube for trichloroacetic acid (TCA) precipitation (for MS)

13 Proteins were precipitated from lysates by the addition of an equal volume of ice-cold 55% TCA.

14 Samples were incubated on ice for 15 min, followed by centrifugation at 21,000 × g for 10 min at 4°C.

15 Protein pellets were resuspended in 1 ml of acetone prechilled to −20 °C and recentrifuged. Pellets were resuspended and recentrifuged another three times in 1 ml of acetone prechilled to −20°C, for a total of four washes

16 Protein pellets were resuspended in Urea Dissolve Buffer (with protease and phosphatase inhibitor) and sonicate for 10 second followed by gentle agitation on an orbital shaker for 1 hr at room temperature.

17 Streptavidin magnetic beads (Thermo Fisher #88817) were resuspended and washed three times in Urea Detergent Wash Buffer for 10 min at 4°C. After washing, streptavidin beads were resuspended in ice-cold Urea Detergent Wash Buffer and 50 µl containing 0.5 mg of beads was added to each sample.

18 Proteins were incubated with streptavidin beads overnight on a rotor at 4°C for 14-18hrs.

19 Beads were washed three times for 5–10 min in 1 ml of Urea Detergent Wash Buffer at room temperature. After the third wash, beads were resuspended in 1 ml of Urea Wash Buffer and transferred to a new tube

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20 After three 5–10 min washes in 1ml Urea Wash Buffer at room temperature, beads were resuspended in 200 µl of Urea Wash Buffer and transferred to a new tube

21 10 µl aliquot (5%) was transferred to a separate tube for western blotting, and the remaining 190 µl of buffer were removed on a magnetic stand. Beads were flash frozen and stored at −80°C

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