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Biotinylation and Purification of Plasma Membrane-associated Proteins from Rodent Cultured Neurons

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[Abstract] This protocol aims at the biotin labeling and affinity purification of plasma membrane proteins from cultured neurons. Protein biotinylation consists in the covalent attachment of biotin to proteins. Biotin is a membrane unpermeable molecule with a small size (MW 244.31 g/mol) and therefore does not interfere with the normal function of proteins. Biotin binds to streptavidin and avidin molecules with high affinity. This binding is extremely resistant to temperature, pH and proteolysis, which allows capture and purification of plasma membrane proteins. Moreover, proteins can bind several biotin molecules, that will allow the consequent binding of several streptavidin or avidin molecules, increasing the sensitivity of detection of the proteins of interest. In this protocol proteins at the cell surface of live cultured neurons are biotinylated. Neuronal extracts are prepared and biotinylated proteins are collected with NeutrAvidin-coupled beads, and analyzed by Western blotting.

Material and Reagents

1. Centrifuge microtube with 0.45 µm filter (VWR International, catalog number: 82031-360)
2. Cell scraper with 1.7 cm blade length (SARSTEDT AG & Co, catalog number: 831830)
3. Primary cultures of rodent hippocampal or cortical neurons (as described in Caldeira *et al*., 2007a; Caldeira *et al*., 2007b; Ferreira *et al*., 2015)
4. Glycine (Sigma-Aldrich, catalog number: 15527)
5. NeutrAvidin plus ultralink resin (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 53151)
6. EZ link sulfo-nhs-ss-biotin (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 21331)
7. Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, catalog number: P7626-1 g)
8. Chymostatin (Sigma-Aldrich, catalog number: C7268-1 mg)
9. Leupeptin (Sigma-Aldrich, catalog number: L2884-1 mg)
10. Antipain dihydrochloride from microbial source (Sigma-Aldrich, catalog number: A6191-1 mg)
11. Pepstatin A (Sigma-Aldrich, catalog number: P5318-5 mg)

12. Deoxycholic acid (DOC) (Sigma-Aldrich, catalog number: D5670)
    Note: it is also named "Sodium deoxycholate monohydrate" on Sigma-Aldrich website.
13. Dithiothreitol (DTT) (NZYTech, catalog number: MB03101)
14. Liquid Nitrogen
15. DMSO (Sigma-Aldrich, catalog number: D8418-100 ml)
16. Pierce™ Bicinchoninic acid protein assay-Reagent A (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 23223)
17. Pierce™ Bicinchoninic acid protein assay-Reagent B (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 23224)
18. NaCl
19. KCl
20. KH_2PO_4
21. Na_2HPO_4
22. $MgCl_2$
23. $CaCl_2$
24. Tris-HCl
25. EGTA
26. EDTA
27. Triton X-100
28. SDS
29. Sodium orthovanadate
30. Bromophenol blue
31. PBS (see Recipes)
32. PBS/Ca^{2+}/Mg^{2+} (see Recipes)
33. Radioimmune precipitation assay (RIPA) buffer (see Recipes)
34. Lysis buffer for insoluble proteins (see Recipes)
35. 2x denaturating buffer (see Recipes)
36. Protease inhibitor cocktail (see Recipes)

Equipment

1. Orbital horizontal shaker (GFL, model: 3005)
2. Sonicator (Sonics & Materials, model: VC50)
3. Benchtop microcentrifuge (Eppendorf AG, model: 5415D)
4. Laboratory tube rotator (Falc Intruments s.r.l., model: F205)
5. Heating block (Bibby Scientific, Stuart Scientific, model: SHT200D)

Procedure

1. Wash cultured cells (primary cultures of rat or mouse hippocampal or cortical neurons at 91.6 x 10^3 cells/cm^2) twice at 4 °C with PBS/Ca^{2+}/Mg^{2+} (3 ml per 60 mm Petri dishes or 2 ml per well of a 6-multiwell plate), in non-sterile conditions.
2. Incubate cells with PBS/Ca^{2+}/Mg^{2+} containing 0.3 to 1 mg/ml EZ Link Sulfo-NHS-SS-Biotin (adjust accordingly to the specificity of your signal on the biotinylated fraction at the final Western blot) for 45 min at 4 °C under mild shaking on an orbital horizontal shaker, in the dark (2 ml per 60 mm Petri dishes or 1.5 ml per well of a 6 multiwell plate).
3. Remove the non-bound biotin by washing the cells twice with PBS/Ca^{2+}/Mg^{2+} supplemented with 100 mM glycine, followed by a 45 min incubation in this solution at 4 °C (3 ml per 60 mm Petri dishes or 2 ml per well of a 6-multiwell plate).
4. Cells lysis:
   a. For low solubility proteins (cells plated in 60 mm Petri dishes): Use 200 µl of lysis buffer supplemented with protease inhibitor cocktail (1:1,000 diluted) per dish, followed by a 30 min incubation on ice. Scrape the cells and transfer the cell extract to a tube. Briefly sonicate (30 sec on ice). Cellular extracts are then incubated with 1% DOC, pH 9.0, 1 h at 37 °C, centrifuged at 18,000 *x g* for 30 min at 4 °C, and the pellet is discarded.
   b. For soluble proteins (cells plated in a 6-multiwell plate): use 400 µl RIPA buffer per well, supplemented with protease inhibitor cocktail (1:1,000 diluted). Scrape the cells and transfer the cell extract to a tube. Briefly freeze the cellular extract in liquid nitrogen. Cellular extracts are thawed and then centrifuged at 6,500 *x g*, 10 min at 4 °C, and the pellet is discarded.

      Note: Low solubility proteins are difficult to extract with RIPA buffer and the extraction yield is low. Therefore, there is the need to plate higher cell number (thus the use of a 60 mm Petri dish instead of a single well of a 6-multiwell plate), and to optimize the lysis buffer and several protocol steps in order to enhance extraction of low solubility membrane proteins.
5. Quantification of the protein content in the above supernatants using the bicinchoninic acid (BCA) method. Protein concentration varies according to the initial cell culture density and efficiency of the solubilization; ideally protein concentration range from 0.7 to 1.1 mg/ml should be obtained.
6. Save 30 µg of protein for total protein blotting.
7. If more than one sample is analyzed.
   a. For insoluble proteins, dilute the same amount of protein to a final volume of 300 µl of lysis buffer supplemented with 1% DOC.

b. For soluble proteins, equalize sample concentration and volumes with RIPA buffer supplemented with protease inhibitor cocktail.

8. To separate the biotinylated proteins, add NeutrAvidin Plus UltraLink Resin in equal amounts to the samples (80 µl for insoluble proteins and 2.5 µl/10 µg of total protein for soluble proteins) and incubate for 2 h at 4 °C with mild shaking on the laboratory tube rotator.
9. Centrifuge 2,500 *x g*, for 3 min, discard supernatant and wash the beads three times with 400 µl lysis buffer (lysis buffer for insoluble proteins supplemented with 1% DOC or RIPA buffer for soluble proteins). Last centrifugation may go up to 5 min.
10. Elute the samples with 2x denaturating buffer at 95 °C for 5 min in a heating block.
11. Centrifuge the samples at maximum speed into a microtube collector with a 0.45 µm filter. Keep the flow-through which contains the protein fraction. The filter will retain the NeutrAvidin Plus UltraLink Resin.
12. Run the total amount of total and biotinylated fractions collected in a SDS-PAGE, followed by Western blot using an antibody against the protein of interest. Adjust the thickness and percentage of acrylamide in the gel according to the volume of sample to be loaded, and to the size of the protein of interest. Purity of the biotinylated fraction should be tested by blotting against an intracellular protein like actin or tubulin, which should be absent in the biotinylated fraction.

Recipes

1. PBS
   137 mM NaCl
   2.7 mM KCl
   1.8 mM KH_2PO_4
   10 mM Na_2HPO_4
2. PBS/Ca^{2+}/Mg^{2+}
   PBS plus 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$ (pH 7.4)
3. RIPA buffer
   150 mM NaCl
   50 mM Tris-HCl (pH 7.4)
   5 mM EGTA
   1% Triton
   0.5% DOC
   0.1% SDS (pH 7.5)
4. Lysis buffer
   50 mM Tris-HCl (pH 7.5)
   1 mM DTT

5 mM EGTA
10 μM EDTA

5. 2x denaturating buffer
   125 mM Tris (pH 6.8)
   100 mM glycine
   4% SDS
   200 mM dithiothreitol
   40% glycerol
   3 mM sodium orthovanadate
   0.01% bromphenol blue
6. Protease inhibitor cocktail
   0.1 M phenylmethylsulfonyl fluoride (in DMSO)
   CLAP (in DMSO):
   a. 1 mg/ml chymostatin
   b. 1 mg/ml leupeptin
   c. 1 mg/ml antipain
   d. 1 mg/ml pepstatin

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