Supporting information

Optimized method for membrane proteins isolation from cultured cells.

(Hernández-Sánchez et al, 2018).

Technical aid for membrane proteins recovery, following the steps as depicted in Figure 1 of fractionation method and described in the main text

Step1.

For a most successful detachment, do not allow the cell culture to become saturated, a confluence of 85 to 90 % is favorable for a complete disaggregation of cells.

Depending on the cell type, the optimum detachment time may be longer. To reduce this time, after culture medium retirement wash the cells three times with cold PBS-EDTA before incubation with warm PBS-EDTA at 37 °C.

Step 2.

Before freezing, the complete elimination of residual buffer is relevant. Use fine tips to allow the pellet to be free of liquid.

From 7 to 10 seconds in liquid nitrogen is the best choice, to replace it use a bath of CO2 ice-ethanol and optimize time for 60 to 120 seconds.

Step 4.

In the hypertonic lysis do not vortex, maintain a constant movement of aproximately 30 inclinations (in angular shaker) or 30 rounds (in orbital shaker) per minute.

Step 5.

The homogenization of membrane pellet with pipet tips will facilitate the recovery of tube wall attached material, but it is not sufficient for protein extraction. Use the sonicator at 50kHz.

NOTE: For long term storage of protein fractions, supplement the hypotonic, hypertonic or 2DE buffers with a protease inhibitor cocktail (Calbiochem ---).

Complementary experimental procedures.

I. Cell culture.

The cell lines, Madin Darby Can Kidney (MDCK) and Human Microvascular Endothelial Cells (HMEC-1) were obtained from the American Type Culture Collection. The cells were

cultured to a confluence of approximately 90%. The MDCK cells were grown in culture medium UltraMDCK (Lonza, USA) without fetal bovine serum (FBS) and supplemented with antibiotics and antifungal (penicillin 100 IU/ml, streptomycin 100 mg/ml and amphotericin B 10 μg/ml) (In vitro, MEX); the HMEC-1 cells were grown in culture medium MCDB131 without L-Glutamine (Invitrogen, USA) supplemented with antibiotics and antifungal, 10ng/mL Epidermal Growth Factor (EGF), 1 μg/mL Hydrocortisone, 10 mM L-Glutamine and fetal bovine serum (FBS) to a final concentration of 10%. Cells were incubated in a humid chamber at 37 °C with 5% CO_2.

II. Method by solubilization

The protocol for preparation of soluble and membrane fractions of animal tissues at the Nevada Proteomics Center was followed using cultured cells harvested as described in the text for the fractionation method. Briefly, cells were homogenized with a Teflon pistil using approximately 20 manual movements in cold homogenization buffer (250 mM sucrose, 1 mM EDTA and 10 mM Tris-HCl buffer pH 7.2). The homogenate was ultra-sonicated using two pulses of 10 seconds (with 30 seconds between pulses) and centrifuged at 100000 x g for 1 hour at 4 °C, the supernatant contains the soluble proteins. The pellet was resuspended in cold homogenization buffer and centrifuged again at 100000 x g for 1 hour at 4 °C, the supernatant was discarded and the pellet (membrane fraction) was recovered in 2DE compatible buffer (8 M Urea, 1% CHAPS, 0.01% bromophenol blue, 15 mM DTT and 0.2% Ampholytes). The protein concentration was determined with the Bradford reagent.

III. Isoelectric focusing (IEF) - first dimension

The IEF was made using prefabricated linear immobilized pH gradient (IPG) strips (Bio-Rad, USA). The IPG strips of 7 cm pH 3 - 10 and pH 4 - 7 were loaded with 25 μg of proteins (when the polyacrylamide gel was stained with silver) dissolved in 200 μl of rehydration buffer and equilibrated overnight at RT. The next day the IEF was performed on a Protean IEF cell (Bio-Rad, USA) at a speed of 8000 V-h with a final voltage of 4000 V in a fast ramp mode.

IV. 2DE - second dimension

When the run was completed the strips were equilibrated for 10 min of equilibration buffer I (6 M urea, 50 mM Tris-HCl buffer pH 8.8, 30% w/v Glycerol, 2% w/v SDS) with 130 mM DTT. Next they were equilibrated for 10 min of equilibration buffer II (6 M urea, 50 mM Tris-HCl buffer pH 8.8, 30% w/v Glycerol, 2% w/v SDS) with 135 mM iodoacetamide. The strips were then placed on a 12% polyacrylamide gel and sealed with a melted 0.7% w/v agarose solution with 0.03% w/v bromophenol blue. Electrophoresis was performed in running buffer (25 mM Tris, 190 mM glycin, 0.1% SDS) in a Mini-PROTEAN Tetra cell (Bio-Rad, USA) at 200 V until bromophenol blue reached 5/6 of the gel (for about 40 minutes). Gels were silver stained.

V. Silver staining

Gels were fixed overnight in 10% trichloroacetic acid (SIGMA, USA), then the solution was discarded and fixation/stop solution (40% v/v Methanol, 10% v/v Acetic acid) was added and left under constant stirring for 30 min. The gels were then washed fourfold with 50 ml of distilled water and then incubated in a 10% glutaraldehyde solution (SIGMA, USA) for 30 min. The gels were washed again as described above and then immersed in developer solution ($AgNO_3$ 20% w/v in 90 mM NaOH, NH_3OH 3.5 mM) until the spots became visible. The reaction was stopped with fixation/stop solution when the closer spots begin to overlap.

VI. Western blot

After electrophoresis, the acrylamide gels were subjected to a humid vertical transfer to PVDF membranes (Bio-Rad, USA) for one hour at constant current (300 mA). The membranes were blocked for one hour with 5% (w/v) defatted milk powder in Tris buffered saline (TBS) with 0.1% Tween-20 at room temperature, and incubated overnight at 4 °C with primary antibody directed against E-cadherin, ERK-2, p65, IκBα, integrin α5, proton channel M2 of influenza virus (Santa Cruz Biotechnology, USA), PAR-1 (R&D Systems, USA), Rab7 (Cell Signaling Technology, USA) and matrix protein M1 of influenza virus (Abcam, UK). The membranes were then washed three times with TBS/0.1% Tween-20. The membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour and developed using solution of Clarity Max Western ECL Substrate (Bio-Rad, USA) with the aid of a ChemiDoc MP System (Bio-Rad, USA).