Subcellular Fractionation

Spheroplasting

1. Grow up 100 ml culture of cells expressing a tagged form of your gene of interest overnight in YPD or selective media
2. Measure OD600
    - if OD600 is over 1.0, dilute cells back to 0.1 and continue growing
    - if OD600 is 0.4-1.0, harvest cells
    - if OD600 is less than 0.4, continue growing
3. Spin down 40 OD600's of cells in conical tubes, 3 min. at medium speed in table top centrifuge
4. Pour off supe and resuspend pellet in 50 ml spheroplast buffer
5. Spin 3 min. at medium speed in table top centrifuge
6. Pour off supe and wash again
7. Resuspend cells in 5 ml of spheroplast buffer and transfer 15 ml conical tube
8. Add zymolase 100T (US Biological, Cat. No. Z1005) to 300 mg/ml (300 µl of 5 mg/ml zymolase)
9. Incubate 30 min. at 30°C with rocking to spheroplast cells (ie. remove the yeast cell wall)
10. Spin cells for 3 min. at slow speed in table top centrifuge
11. Pour off supernatant
12. Gently resuspend pellet in 5 ml spheroplast buffer by shaking
13. Spin cells for 3 min. at slow speed in table top centrifuge
14. Pour off supernatant and repeat wash 2 more times
15. Freeze spheroplasts in liquid nitrogen
16. Can store spheroplasts at –80°C for up to a month

Lysate preparation and fractionation

1. Chill 5 cm^3 Dounce homogenizer (Bellco Glass Inc., Cat. No. 1984-10015) with A pestle on ice
2. Thaw spheroplasts on ice
3. Resuspend in 2.5 ml of spheroplast lysis buffer
4. Transfer to Dounce homogenizer and lyse cells with approximately 30 strokes on ice (you can check lysis by putting 5 µl of cells before and after Douncing on a slide and looking at them on the microscope)
5. Save 100 µl of lysate=CRUDE EXTRACT (put it in an eppie on ice)
6. Transfer lysate to 2 1.5 ml eppies
7. Spin lysates in microfuge at 13,000 x g for 15 min. at 4°C
8. Remove supe into eppie=S13 (this is your cytoplasmic fraction, but it will also have microsomal membranes)
9. Resuspend each pellet in 1.25 ml of spheroplast lysis buffer by pipetting up and down many times=P13 (it contains the nucleus, rough ER, DNA, etc.)
10. If necessary, save 100 µl of P13 in eppie on ice
11. Transfer remainder of P13 to ultracentrifuge tubes (Beckman Coulter, Cat. No. 355618) and **balance to 0.1 g** using the scale (Make sure the tubes are full with

liquid because they will implode in the ultracentrifuge. Add more buffer to the tubes to fill them.)

12. Centrifuge for 60 min. at 4°C at 70,000 rpm in a Type 70Ti rotor (Beckman Coulter, Cat. No. 337922) in the ultracentrifuge (Beckman Coulter Optima L-100 XP Ultracentrifuge)
13. Remove supernatant following the spin=S100 (this is the cytoplasmic fraction)
14. Resuspend pellets in 2.5 ml total spheroplast lysis buffer by pipetting up and down many times=P100 (this has the microsome membranes)
15. If your protein is abundant, remove 100 μl of each fraction (CRUDE, S13, P13, S100, P100) into new eppie containing 50 μl 4X SDS sample buffer containing beta-mercaptoethanol
16. Poke holes in top of eppies with needle
17. Incubate samples for 5 min. at 100°C in the sand bath
18. Load 10-20 μl onto SDS-PAGE; don't forget to load extra lanes for control blots and remember the markers (load CRUDE, S13, P13, S100, P100)
19. Transfer to PVDF (Millipore, Cat. No. IPVH07850) and do western blots—to detect your protein of interest, glucose-6-phosphate dehydrogenase (50 kDa) as a cytoplasmic protein, Pom152 as a membrane protein (152 kDa) or Mps3 (98 kDa)

Membrane extraction

1. Make lysates and fractionate to generate P13 (through step 9 above)
2. Aliquot 300 μl P13 into 7 tubes
3. Spin each in microcentrifuge at 13,000 x g for 15 min. at 4°C
4. Aspirate off supernatant
5. Resuspend one tube in each of the following by vigorously pipetting:
    A. 500 μl spheroplast lysis buffer
    B. 500 μl spheroplast lysis buffer containing 10 mM EDTA
    C. 500 μl spheroplast lysis buffer containing 1M NaCl
    D. 500 μl spheroplast lysis buffer containing 0.2M Na_2CO_3 pH 11
    E. 500 μl spheroplast lysis buffer containing 6M urea
    F. 500 μl spheroplast lysis buffer 1% TritonX-100
    G. 500 μl spheroplast lysis buffer 1% TritonX-100/1M NaCl
6. Incubate 15 min. on ice
7. Spin each in microcentrifuge at 13,000 x g for 15 min. at 4°C
8. Remove supe from each into new eppie containing 100 μl of 4X SDS sample buffer (this is your S)
9. Resuspend each pellet in 300 μl of spheroplast lysis buffer by vigorous pipetting
10. Add 100 μl of 4X SDS sample buffer to the resuspended pellets (this is your P)
11. Poke holes in top of eppies with needle
12. Incubate samples for 5 min. at 100°C in the sand bath
13. Load 10-20 μl onto SDS-PAGE (load as SA, PA, SB, PB, SC…etc.)
14. Transfer to PVDF and do western blots to detect your protein of interest (I generally don't bother with the controls at this point, but you can do Mps3 and compare it to Jaspersen et al. 2002—Mps3, like other integral membrane proteins, will only be extracted and appear in the S when TritonX-100 is present—

peripheral membrane proteins are generally extracted with sodium carbonate and urea, sometimes salt)

Spheroplast Buffer (store at 4°C)
50 mM Tris-HCl, pH 7.5
1.2 M sorbitol
10 mM NaN3
40 mM β-mercaptoethanol (added fresh)

2X Spheroplast Lysis Buffer (store at 4°C)
40 mM HEPES/KOH, pH 7.4
200 mM K-acetate
10 mM Mg-acetate
2 mM EDTA

Add 1 complete protease inhibitor tab/ 50 ml buffer and 2 mM PMSF and 2 mM DTT, fresh on the day of the experiment