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PROCEDURE FOR PREPARING VACUOLAR MEMBRANES AND OTHER MEMBRANE FRACTIONS FROM NEUROSPORA CRASSA (4 liter prep, easily scaled up to 10, 12, 24, or 30 l)

CONIDIAL FLASKS: Prepare one conidial flask for 4 l (or up to 10 l) of medium. A conidial flask has 80 ml of solid medium in a 500 ml Erlenmeyer flask. We use strain 74A as the wild type. After inoculating the conidial flask, place it in the 30˚C incubator for 3 days, then at 25°C in the light for the next 3-7 days. It is best to harvest the conidial flasks at 7-11 days. The conidial flasks can be stored at -20°C for two weeks or so. With time, conidia become inviable.

MEDIA PREPARATION: One or two days before you actually prepare vacuolar membranes, autoclave the growth medium, harvesting apparatus, water for harvesting conidia, and a graduated cylinder for measuring the inoculum.

For a 4 liter prep:

4 liters of Vogel's Minimal Medium with 2% sucrose in a 6 l Florence flask (or a glass carboy). A bubbler consisting of a glass rod, reaching close to the bottom of the growth flask, inserted into a two-holed rubber stopper. Autoclave separately, wrapped in foil. (Otherwise, medium tends to spill.)

500 or 1000 ml Erlenmeyer flask with distilled water for harvesting conidia. 100 or 250 ml graduated cylinder.

1 500 ml Erlenmeyer flask containing a cup of 4 layers of cheesecloth or a vacuum flask fitted with a plastic filter funnel with 2 (or 4) layers of cheesecloth for harvesting conidia. Secure the cheesecloth with a rubber band and cap the flask with foil.

Autoclave all of the above items for 30-40 minutes. Allow the medium to cool to room temperature, which can take several hours. When the carboys are cooled, place them in the 25°C water bath.

HARVESTING CONIDIA: Using sterile technique, pour approx. 100 ml of sterile water into the conidial flask. Re-cap and shake the flask to dislodge the conidia. Pour the conidial suspension through the cheesecloth-covered flask (or filter funnel using vacuum). Repeat with a second 100 ml of water. After harvesting, discard the cheesecloth and re-cap the flask with foil.

COUNTING CONIDIA: Use a hemacytometer to determine the number of conidia per ml of suspension. Make a 1:100 dilution of your conidial suspension in distilled water. Count the number of conidia in the four large squares (with 4 x 4 smaller internal squares) in the outside corners of the grid on the hemacytometer. Each large square has a volume of 10^{-4} ml. Calculate the number of conidia/ml of your conidial suspension (number of conidia/large square x 10^6).

INOCULATING THE CULTURES: Depending on what time you want to begin your prep in the morning (allow 30-45 minutes to get everything set up before you actually harvest the cells), inoculate the cultures the evening before allowing for 14.5 - 16 hours of growth time. Inoculate the cultures with 10^6 conidia per ml of medium. After inoculation, aerate the cultures by bubbling air through the medium. (We are now using an air pump, because the building air supply was causing the cultures to become "slimy." Proper filters might prevent this problem.) This is also a good time to check the water bath temperature, which should be 26°C.

SOLUTIONS NEEDED: It is best to check to make sure the following solutions are in adequate supply at least one day before you plan to do the prep.

VPM (Vacuolar Preparation Medium, stored in the cold room) (10 mM HEPES, 1 mM Na2EDTA.2H20, 1 M sorbitol, pH adjusted to 7.5 with 10N NaOH).
(You need approx. 1.0 liter for a 4 l prep.)

100 mM Na2ATP, ph adjusted to 7.5 with Tris base (solid first, then 0.5 M)

1 mM Na2EDTA, pH adjusted to 7.5 with Tris base. Use to make ECA (next).
(Can also use EGTA, much harder to dissolve. Takes about 30 min to get pH stable. We used this for PM preps in the old days.)

ECA (1 mM Na2EDTA, 2 mM Na2ATP, 2 ug/ml chymostatin, pH 7.5)

100 mM PMSF (phenylmethylsulfonylfluoride) in 100% ethanol. (This is a toxic solution. Wear gloves.) (174 mg/10 ml)

Chymostatin in DMSO (20 mg/ml stock solution)

30%, 40% and 50% Sucrose Mixes (1 mM Na2EDTA, 10 mM HEPES, sucrose, 2mM ATP, 2 ug/ml chymostatin)

2 Sucrose step gradients in 15 ml polycarbonate Sorvall tubes with jackets. Prepare the day before use. Add 7 ml 50% Sucrose Mix, 2 ml 40% Sucrose Mix, and 2 ml 30% Sucrose Mix. Store at 4 oC.

The VPM and sucrose gradients are stored at 4 oC. The other solutions are stored in the -20°C freezer. Allow time for the ATP and ECA solutions to thaw and place them on ice.

ROTORS AND CENTRIFUGES: all precooled at 4 ºC

Beckman JA14 rotor for Beckman J2-21M centrifuge (or Sorvall GSA rotor for Sorvall RC-5B superspeed centrifuge)
Beckman JA20 rotor for Beckman J2-21M centrifuge (or Sorvall SS34 rotor)
Beckman TLA100.3 for Beckman Tabletop Ultra-centrifuge

PRE-PREP SET-UP: After the bubbling cultures have grown overnight, check the dry weight. Collect 25 ml of cells, vacuum filter them through Whatman #1 or #540 filter paper, suck dry, rinse with a small amount of distilled water, wash with acetone to remove water, and weigh the pad. The optimum dry weight at which to harvest the cultures is 0.8 – 1.2 mg/ml. For example, .025 grams per 25 ml gives a dry weight of 1.0 mg/ml.

You will also need two large and two small containers of ice. Chill 2 GSA bottles, 1 liter of VPM, the ATP, ECA, and PMSF. Put 170 ml of glass beads in the bead-beater container, rinse the beads 7-8 x with VPM, fill 3/4 full with VPM (allowing room for cells), and place the container in ice. Just prior to harvesting the cultures, add 2 mls 100 mM Na_2ATP, 2 mls 100 mM PMSF, and 20 ul chymostatin to each bead-beater container with glass beads. Mix 70 ml VPM, 0.7 ml ATP, and 7 ul chymostatin for rinsing the beads; chill. (Note: can also rinse the beads by transferring them to a sintered glass funnel and sucking the VPM throug. Improrant to be thorough!).

Set up a harvesting flask - a large plastic filter funnel with 6 layers of cheesecloth on a glass carboy in the sink.

As various membrane fractions are isolated, suspend them in the appropriate solution, aliquot to microfuge tubes, freeze in liquid nitrogen, and store in a –80 oC freezer.

HARVESTING: Vacuum filter the 4 l culture and rinse the cells with a small amount of cold distilled H2O. Squeeze out excess water from the cells and place them in the chilled bead-beater container. Add enough VPM to fill the chamber with liquid during the bead-beating (avoid air bubbles). Beat for 1 min. and 30 sec. Pour off the homogenate into a GSA bottle. Rinse the beads 2x with 35 ml VPM/ATP/chy. Add rinsate to homogenate.

CENTRIFUGATIONS:

#1. JA14 rotor in Beckmanl centrifuge at 4°C.
Speed: 2500 rpm (approx. 1,000 x g).
Time: 10 minutes
Save the supernates and discard the pellets (cell wall, nuclei, glass beads). Pour off the
supernatants into 2 pre-chilled GSA bottles.

#2. JA14 rotor in Beckman centrifuge at 4°C.
Speed: 10,000 rpm (approx. 15,000 x g).
Time: 30 minutes
Carefully pour off the supernatant, keeping the pellet down, and save for isolation of plasma membranes and other light membranes. The pellet containing mitochondria and vacuoles will be soft and runny. (Note: This fraction is largely mitochondria and can be used as a mitochondrial fraction for many purposes.)

Place the bottles on ice with the pellet down and suction off any extra sup and loose mitochondrial slurry. Gently resuspend the pellets with 0.5 ml VPM/ATP/Chymostatin (Prepare 10 ml VPM, 100 ul 0.1 M Na2ATP, 1 ul 2% chymostatin), using a small pestle and a cut-off blue pipette tip. Pool the resuspended pellets in a chilled glass Dounce homogenizing vessel. Rinse each bottle with 0.25 ml VPM/ATP/ Chymostatin and bring the total volume of the resuspended pellets to 4 mls. Homogenize by hand with 3-4 strokes of the pestle. Layer the homogenate onto two sucrose step gradients. The gradients will separate mitochondria and vacuoles.

#3. JA20 rotor in Beckman centrifuge at 4 oC
Speed: 19000 rpm (abut 46,000 x g)
Time: 60 min

PLASMA MEMBRANE ISOLATION

The other spaces in the rotor can be used to pellet the plasma membranes at the same time. Fill 6 40 ml tubes with supernatant from centrifugation #2 and centrifuge as for #3. Save the supernatant to collect "light" membranes – mostly ER (?), Golgi, VM from broken vacuoles, etc. Suspend the pellets containing plasma membranes in 40 ml total of 1 mM EGTA/Tris to pH 7.5, using a Pasteur pipette. Transfer the membrane vesicles to a 55 ml Dounce homogenizer. Homogenize by hand with 5-6 strokes, or use a motor-driven pestle with 10 strokes at rather high speed. (Both seem to work fine.) Put water in balance tube.

#3' JA20 rotor in Beckman centrifuge at 4 oC
Speed: 19,000 rpm
Time: 40 min

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Suspend the pellet (plasma membrane fraction) in 0.5-1.0 ml ECA with a small pestle and pipette tip. Transfer to a small homogenizer and blend with several strokes. Store in aliquots at –80 oC.

VACUOLAR MEMBRANE ISOLATION (and mitochondria)

Suction off the sucrose and everything above the vacuolar pellet. (Collect gradient purified mitochondria, the obvious huge brown band, with a Pasteur pipette if you wish and store at –80

oC.) Use a Q-tip or kimwipe and forceps to wipe out the inside of the tube to remove any remaining material that cannot be suctioned off (careful not to disturb the pellet). This will reduce the amount of mitochondrial contamination in your final VMs.

Lyse the vacuoles by resuspending each pellet in 0.5 ml ECA/PMSF (13 ml ECA, 0.13 ml PMSF) using a pestle and Pasteur pipette. Pool the resuspended pellets in a chilled glass homogenizing vessel. Rinse each tube with 0.5 ml ECA, and bring the total volume of the resuspended pellets to 6.0 mls. Use the teflon homogenizer pestle (3-4 smooth passes) to blend the mixture and distribute the homogenate into 2 pre-chilled TLA 100.3 tubes (3 ml per tube).

#4. TLA100.3 rotor in Beckman TLA100 Ultracentrifuge (4°C).
Speed: 20,000 rpm (approx. 20,000 x g)
Time: 5 minutes
(Pellets contain organelles with Woronin bodies and any remaining broken glass, mitos, and cell wall.)

Using a Pasteur pipette and being careful not to disturb the pellets, remove the supernatants and transfer them to two new pre-chilled TLA100.3 tubes. Re-balance the pair.

#5. TLA100.3 rotor in Beckman TLA100 Ultracentrifuge (4°C).
Speed: 60,000 rpm (approx. 195,000 x g)
Time: 10 minutes

Remove the supernatants (vacuolar contents) and save if desired. Resuspend the pellets vacuolar membranes) in 3.0 ml ECA total. Balance against water.

#6. TLA100.3 rotor in Beckman TLA100 Ultracentrifuge (4°C).
Speed: 55,000 rpm (approx. 164,000 x g)
Time: 10 minutes

Discard the supernatants and resuspend the vacuolar membrane pellet in 150-300 ul ECA. Transfer to a small homogenizer, mix evenly, and distribute into aliquots of about 50 ul per tube plus one 30 ul aliquot for initial assays of protein and V-ATPase activity). Freeze sample in liquid nitrogen and store at -80°C (really –70 oC for us).

ISOLATION OF ER AND OTHER LIGHT MEMBRANES

The supernatant from the tubes used for pelleting PM in #3 can be used to isolate a light membrane fraction. Dispense the supernatant to quick seal ultracentrifuge tubes.

#7: Beckman Ti60 rotor (angle) in a Beckman ultracentrifuge
Speed: 40,000 rpm (114,000 x g_{av})
Time: 60 min

Suspend ER pellets (rather loose) in 0.8-1.0 ml ECA and store frozen in aliquots, or wash them right away by diluting in 3 ml in ECA and centrifuging.

#8: TLA100.3 rotor in Beckman tabletop ultracentrifuge
Speed: 70,000 rpm (265,000 x g)
Time: 30 min

Suspend washed ER pellets (now firm) in about 200 ul ECA. Homogenize. Freeze in liquid N2. Store in aliquots at –80oC.