Video Article

Isolation and Respiratory Measurements of Mitochondria from *Arabidopsis thaliana*

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Abstract

Mitochondria are essential organelles involved in numerous metabolic pathways in plants, most notably the production of adenosine triphosphate (ATP) from the oxidation of reduced compounds such as nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂). The complete annotation of the *Arabidopsis thaliana* genome has established it as the most widely used plant model system, and thus the need to purify mitochondria from a variety of organs (leaf, root, or flower) is necessary to fully utilize the tools that are now available for *Arabidopsis* to study mitochondrial biology. Mitochondria are isolated by homogenization of the tissue using a variety of approaches, followed by a series of differential centrifugation steps producing a crude mitochondrial pellet that is further purified using continuous colloidal density gradient centrifugation. The colloidal density material is subsequently removed by multiple centrifugation steps. Starting from 100 g of fresh leaf tissue, 2 - 3 mg of mitochondria can be routinely obtained. Respiratory experiments on these mitochondria display typical rates of 100 - 250 nmol O₂ min⁻¹ mg total mitochondrial protein⁻¹ (NADH-dependent rate) with the ability to use various substrates and inhibitors to determine which substrates are being oxidized and the capacity of the alternative and cytochrome terminal oxidases. This protocol describes an isolation method of mitochondria from *Arabidopsis thaliana* leaves using continuous colloidal density gradients and an efficient respiratory measurements of purified plant mitochondria.

Video Link

The video component of this article can be found at <https://www.jove.com/video/56627/>

Introduction

The history of plant mitochondrial research goes back over 100 years¹. Intact mitochondria were first isolated in the early 1950s using differential centrifugation. The advent of a colloidal density gradient in the 1980s allowed mitochondria to be purified without suffering osmotic adjustment. While gradient purified mitochondria are suitable for most purposes, due to the sensitivity of mass spectrometry, even relatively minor contaminants can be detected and may be inappropriately assigned a mitochondrial location². The use of free flow electrophoresis can remove both plastidic and peroxisome contamination³, but free flow electrophoresis is a highly specialized technique and is not required for the vast majority of studies. Furthermore, when determining the location of a protein it needs to be remembered that dual or multiple targeting of proteins occurs in cells. Over 100 dual targeted proteins are described for chloroplasts/plastids and mitochondria⁴, and a number of proteins targeted to mitochondria and peroxisomes are also known⁵. Furthermore, the re-location of proteins under specific stimuli, e.g. oxidative stress, is an emerging theme in cell biology⁶. Thus, the location of proteins needs to be considered in the context of the biology studied, and a variety of approaches are used to determine and verify location².

Mitochondria are typically isolated from plant tissues by homogenization, a balance is required between breaking open the cell wall to release mitochondria, and not damaging the mitochondria. Traditionally, with potato and cauliflower, homogenization involves using household blender/juicer apparatus to make a liquid extract in a buffer with various components to maintain activity. Isolation of mitochondria from pea leaves, (a popular material for mitochondrial isolation using young seedlings (~10 days old), utilizes a blender to lyse cells as the leaf material is soft. With the availability of *Arabidopsis thaliana* T-DNA insertional knock-out lines, the need to be able to purify mitochondria to carry out functional studies has necessitated the development of methods to isolate mitochondria from leaf, root or flower tissue. Overall the methods developed for other plants worked well⁷, with the perquisite that grinding of the material needed to be optimized. For *Arabidopsis* this can be achieved in a variety of ways (see below), and differs between tissue types (root versus shoot). The use of the continuous gradient can also be optimized as the density of mitochondria from different organs or developmental stages means they can migrate differently. Thus, for maximum separation the density of the gradient can be refined to ensure to achieve best separation.

Once purified the mitochondria can be used for a variety of studies, including protein and tRNA uptake experiments, enzyme activity assays, respiratory chain measurements and western blot analyses. Isolated mitochondria can also be used for mass spectrometry analyses of protein abundance. Targeted multiple reaction monitoring (MRM) analyses allows for the quantification of defined proteins, but require significant assay development. In contrast, quantification by dimethyl or other isotope labels⁸, provides a discovery approach in identifying differences across the whole proteome that can be used to uncover novel biological insights.

Protocol

This protocol is used for the isolation of intact mitochondria from *Arabidopsis thaliana* organs grown on soil using continuous colloidal density gradients. All procedures following the collection of the material are carried out at 4 °C.

1. Preparation of Grinding Medium, Wash Buffer, and Gradient Solutions

1. Prepare 300 mL of grinding medium (minus ascorbate and cysteine) and 200 mL of 2x wash buffer per **Table 1** one day prior to the isolation, keep them cold at 4 °C.
NOTE: Add sodium ascorbate and L-cysteine (free base) to a final concentration of 17.84 mM and 20.36 mM, respectively, to the grinding medium on the morning of the isolation. If the mitochondria are to be used for western blot or blue native-polyacrylamide gel electrophoresis (BN-PAGE) analyses, make up 2x wash buffer without BSA.
2. Prepare gradients on the morning of mitochondrial isolation (**Figure 1** and **Table 1**).
 1. Make the heavy and light gradient solutions in two beakers; 35 mL of each is sufficient for two gradient tubes.
 2. Wash the chambers of the gradient pourer and PVC peristaltic tubing with deionized water and ensure even flow through the tubing from all tubing.
 3. Place 2 centrifuge tubes (50 mL) at a slight angle on ice with the PVC peristaltic tubing outlets taped to the inside of the tubes to ensure the gradient solution runs down the side of the tubes.
 4. Close the connection between the inner and outer chambers (black lever down). Place gradient pourer on a magnetic stirrer with a small stir bar in the inner chamber.
 5. Pour the 35 mL heavy gradient solution into the inner chamber (chamber with tubing outlet). Dispense the heavy gradient solution into the two centrifuge tubes placed on ice until half is remaining with the peristaltic pump rate set to fast (300 mL/h).
 6. Pour the 35 mL light gradient solution into the outer chamber (chamber without tubing outlet). Open the connection between the chambers (push black lever up halfway) and allow solutions to mix gently. Mix the solutions using the magnetic stirrer.
 7. Allow the solutions to run slowly (60 mL/h) until all of the gradient mix has been dispensed from the chambers resulting in two 0 - 4.4 % (w/v) polyvinylpyrrolidone (PVP) gradient-filled tubes. Keep the gradients on ice until ready to use in Step 2.12.
NOTE: Once the gradient has been prepared, dilute 2x wash medium to 1x with prechilled-deionized water and keep cold at 4 °C.

2. Homogenization and Mitochondrial Isolation

1. Cut whole rosette tissue from 4-week old *Arabidopsis* plants grown on soil with scissors, at least 80 plants will be required for 1 prep.
NOTE: 10 - 14 day old water cultures, minimum 5 pots/prep, or 2-week old seedlings grown on Murashige & Skoog Basal Salt Mixture (MS) agar plates, minimum of 4 plates, may also be used.
2. Place half of the plant material that has been cut into small pieces in a pre-cooled 4 °C large mortar and pestle with 75 mL of grinding medium (**Table 1**), and grind extensively for several minutes until no big pieces of tissue are left.
3. Pre-wet 4 layers of filtration material (22 - 25 µm pore size) with grinding medium and filter homogenate through the 4 layers of filtration material through a plastic funnel into a 500 mL conical flask.
4. Grind the remaining half of the tissue with another 75 mL of grinding medium.
5. Combine homogenates in the filtration materials and filter as much as possible homogenate through.
6. Grind the remaining tissue remaining on the filtration material again with the remaining 150 mL of grinding medium, filter again into the 500 mL conical flask.
7. Centrifuge the filtered homogenate in 8 pre-chilled 50 mL plastic centrifuge tubes at 2,500 x g at 4 °C in a fixed angle rotor for 5 min.
8. Pour the supernatant into clean centrifuge tubes (50 mL; avoid disturbing the green pellet) and centrifuge at 17,500 x g at 4 °C for 20 min in a fixed angle rotor.
9. Discard all but <3 mL of the supernatant by aspiration (or gently pour off without disturbing the pellet). Gently resuspend the pellet in the residual supernatant using a small fine paintbrush.
10. Add 10 mL of 1x wash buffer to each tube and combine 4 tubes into 1 clean centrifuge tube (i.e., resulting in 2 tubes).
11. Fill these tubes with 1x wash buffer to 50 mL and repeat steps 2.7 - 2.9.
12. Pool crude mitochondrial pellets into 1 tube using a dropper and disperse evenly with the fine paintbrush (a small amount of wash buffer may be used but the final volume needs to be less than 3 mL to fit on top of the gradient). Gently layer the mitochondrial suspension with a dropper onto the 2 continuous 0 - 4.4 % (w/v) PVP gradients.
13. Balance tubes by weight (using 1x wash buffer) and centrifuge at 40,000 x g at 4 °C for 40 min. Ensure the break is turned off. Mitochondria will migrate to a band near the bottom of the tube, or may be present in the heavy solution at the bottom of the tube (identified by a cloudy, yellowish band; **Figure 2**).
14. Carefully remove the upper 5 cm of solution above the mitochondrial band by aspiration.
15. Distribute the remaining solution containing the mitochondria into 2 clean tubes and fill with 1x wash buffer. Evenly distribute the colloidal density gradient and mitochondria by covering the mouth of the tube with a plastic paraffin film and inverting several times. Centrifuge for 15 min at 31,000 x g at 4 °C with slow braking.
NOTE: Before centrifugation, make sure the mitochondria and the remaining colloidal density gradient are evenly distributed. Otherwise, the colloidal density gradient may concentrate at the bottom of the tube and prevent pelleting of the mitochondria.

16. Remove the supernatant by aspiration, fill the tube with 1x wash buffer up to 50 mL, and invert again to ensure mixing. Centrifuge for 15 min at 31,000 x g at 4 °C with slow braking.
17. Aspirate the supernatant and collect the mitochondrial pellets in as small a volume (~500 µL) as possible using a pipette with modified 200 µL tips (cut the tip a little above its end to increase its opening). Place the mitochondria into a 1.5 mL tube and keep on ice for immediate use or store at -80 °C.
NOTE: If mitochondria will be used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), BN-PAGE, or immunoblot analyses, use 1x wash buffer without BSA for the final washes (*i.e.*, step 2.15 and 2.16).
18. Determine the protein concentration of mitochondria using the Bradford method.
NOTE: For BN-PAGE centrifuge aliquots at 4 °C, and store pellet at -80 °C until use. For oxygen consumption measurements, only freshly isolated mitochondria can be used.

3. Oxygen Consumption Measurements

NOTE: Oxygen consumption by freshly isolated plant mitochondria can be analyzed with a Clark-type oxygen electrode, enabling the determination of mitochondrial intactness, cytochrome c pathway activity, and alternative pathway activity.

1. Software installation
 1. Install licensed oxygen monitoring software onto the computer used for oxygen consumption measurements.
2. Preparation of respiration medium, substrates, chemical stock solutions, and the Clark-type oxygen electrode
 1. Prepare respiration medium (300 mM sucrose, 10 mM NaCl, 5 mM KH₂PO₄, 2 mM MgSO₄, 0.1 % (w/v) bovine serum albumin (BSA), 10 mM TES (pH 7.2)) used for oxygen consumption assays.
 2. Prepare substrates, inhibitors, and effectors used for measuring mitochondrial respiration (**Table 2**). These can be prepared earlier and stored at -20 °C.
NOTE: Neutralize the pH of acidic solutions such as organic acids to pH 7.0 using NaOH before use.
 3. Warm the respiration medium to the same temperature as the assay chamber (25 °C).
 4. Assemble the Clark-type oxygen electrode as described in the protocol of the manufacturer.
 5. Place 1 mL air-saturated water (air-saturated water is obtained by vigorously shaking a small quantity of deionized water in a large conical flask) in the measuring chamber. To switch on the stirrer, click the "Stirrer Speed" button. Click the "On" button and set the stirrer speed to 65 rpm. Click "OK" to continue. Stir the water continuously at 25 °C.
3. Calibration of oxygen electrode
 1. Warm the water that will be used for calibration to the same temperature as the assay chamber (25 °C).
 2. Wait for the current to stabilize and calibrate the oxygen electrode.
 3. To start calibration of the oxygen electrode, click the "Calibrate" button, select "Liquid Phase Calibration," and then "Air Saturated Water."
 4. A new window will open (Oxygen Calibration (Liquid Phase) - Step 1 of 5). Select the channel to calibrate (the channel the electrode control box is attached to) and enter variables (set "Chamber Temperature" to 25 °C and "Atmospheric Pressure" to 101.32 kPa). Click "OK" to continue.
 5. In the next step (Step 2 of 5), setup the stirrer speed (65 rpm) and click "OK" to continue.
 6. In step 3 of 5, wait for the signal to reach a plateau. The term "Plateau reached, press OK to continue" will appear in the box. Click "OK" to continue.
 7. In step 4 of 5, establish zero oxygen in the chamber by adding 5 mg sodium dithionite. Click "OK" to continue.
NOTE: A decline in oxygen concentration should be visible in the trace and should reach 0.
 8. In the last step of calibration (Step 5 of 5), wait for the signal to reach a plateau. The term "Plateau reached, press OK to continue" will appear in the box. Click "OK" to continue.
 9. Click "Save Calibration" to save the calibration. A new window will open. Click "OK" to confirm to save the new calibration for the oxygen control box.
NOTE: After successful calibration, the labeling "Oxygen (nmol/mL)" will appear on the y-axis.
 10. After calibration, clean the measuring chamber by rinsing the chamber with water (at least 5 - 10 times) to ensure proper cleaning. Place 1 mL of respiration medium into the measuring chamber and allow the membrane to equilibrate for a few minutes until the oxygen consumption trace is linear.
 11. Adapt the scale of the oxygen consumption trace to get a good slope view. Click "Zoom XY" and enter variables (set "Oxygen" to 0 - 300 and the "Time Axis" to 0 - 45 min).
4. Determination of mitochondrial integrity
 1. With plunger open, add 970 µL air-saturated respiration medium to the reaction chamber.
 2. Add 30 µL isolated mitochondria or 150 µg of total mitochondrial protein as determined after mitochondria isolation (total protein needs to be included in calculations afterwards) to the reaction chamber using a pipette with a cut tip.
 3. Stir the mitochondrial suspension continuously using a magnetic stirrer bar (65 rpm) at 25 °C to air-saturate the solution.
 4. Seal the chamber with the plunger, click "Start Recording" to record oxygen consumption, and allow the oxygen consumption trace to stabilize (1 - 2 min).
 5. Determine the rate of oxygen consumption manually after adding 10 mM ascorbate and 25 µM cytochrome c for 5 min to get the "before detergent" rate.
NOTE: To label the addition of substrates, inhibitors, and effectors directly in the trace, click the "Add Event Mark" button and enter the corresponding label designation. Click "OK" to continue.
 6. Determine the rate of oxygen consumption manually for 3 min after adding 0.05% (v/v) detergent to give the "after detergent" rate. Click "Stop Recording."
 7. Click the "Rates of Change Table" button. 2 cursors will appear as vertical lines on the graph screen. Click and drag the pair of rate cursors to the required positions in the trace to define the rate interval. Move the rate interval along the trace using the left rate cursor.

Set the rate interval itself using the right rate cursor. The oxygen monitoring software automatically draws a line of best fit between the two cursors while moving the rate interval cursors along the trace.

8. Once the desired rate interval and position has been defined, enter the rate into the table with a right click on 1 of the 2 cursors, and select "Add Rate to Table." Click the "Add" button to confirm to display the rate on the main rate table.
9. Calculate mitochondrial integrity by dividing the "before detergent" rate by the "after detergent" rate, expressed as a percentage, and subtract it from 100.

5. Determination of mitochondrial respiration

1. With plunger open, add 970 μ L of air-saturated respiration medium to the reaction chamber. Add 500 μ M ATP and 30 μ L isolated mitochondria or 150 μ g of total mitochondrial protein using a pipette with a cut tip to the respiration medium in the reaction chamber.
2. Stir the mitochondrial suspension continuously using a magnetic stirrer bar (65 rpm) at 25 $^{\circ}$ C.
3. Close plunger, click "Start Recording" to record oxygen consumption, and wait for 1 - 2 min for oxygen consumption rate to stabilize (when the oxygen consumption trace is linear). Add 5 mM succinate. Record the rate of oxygen consumption for about 2 min.
NOTE: To label the addition of substrates, inhibitors, and effectors directly in the trace, click the "Add Event Mark" button and enter the corresponding label designation. Click "OK" to continue.
4. Add 1 mM adenosine diphosphate (ADP). Continue recording the rate of oxygen consumption for 2 min.
5. Add 1 mM NADH. Record the rate of oxygen consumption for 4 - 8 min. This rate is the capacity of respiration via cytochrome oxidase.
6. Add 1 mM potassium cyanide (KCN) (or 2.5 μ M myxothiazol or 5 μ M antimycin A) to inhibit the cytochrome c pathway, and continue to record for about 2 min to observe oxygen consumption via the alternative oxidase.
7. Add 5 mM dithiothreitol (DTT) and 10 mM pyruvate to fully activate the alternative oxidase. Continue to record for 5 - 7 min: This is the capacity of the alternative oxidase.
8. Add 500 μ M n-propyl gallate (nPG), and record for 2 min. This assesses residual oxygen consumption rate that cannot be chemically inhibited. Subtract this rate from the other rates recorded, as it is not likely to be mitochondrial in origin. Oxygen consumption still occurring after inhibition of the cytochrome c and AOX pathway (by KCN and nPG) is very likely to come from peroxidases present as contaminants in the isolated mitochondria⁹.
9. Click "Stop Recording."
10. Click the "Rates of Change Table" button and enter the rate into the table with a right click on one of the two cursors, and select "Add Rate to Table". Click the "Add" button to confirm to display the rate on the main rate table.
NOTE: When adding effectors dissolved in organic solvents (e.g., antimycin A, nPG, myxothiazol), the chamber and plunger must be rinsed with ethanol and then water to ensure proper cleaning.

Representative Results

Using this protocol, we were able to detect different mitochondrial proteins by SDS-PAGE and immunoblotting. As shown in **Figure 3A**, the protein isolated from water culture tissue is sufficient to detect a faint band (2 μ g). Signal intensity increases proportionately to the amounts loaded. For mitochondria isolated from tissues grown on plates (**Figure 3B**), the response to high light stress treatment in different genotypes can be analyzed by immunodetection with alternative oxidase antibodies.

The intactness of mitochondria, cytochrome c pathway activity and alternative pathway can be measured using freshly isolated mitochondrial samples. Mitochondrial integrity is well preserved during the described isolation procedure (**Figure 4A**). Adding different substrates, inhibitors and effectors to isolated mitochondria, oxygen consumption through the cytochrome c pathway and alternative oxidase pathway is influenced (**Figure 4B**).

Figure 1: Setup for the preparation of the gradients. Left: Centrifuge tubes (50 mL) with a slight angle placed on ice with PVC peristaltic tubing outlets taped to the inside of the tubes; Middle: Peristaltic pump; Right: Gradient pourer on top of a magnetic stirrer containing the heavy gradient (inner chamber) and light gradient (outer chamber) solutions. [Please click here to view a larger version of this figure.](#)

Figure 2: Purification of mitochondria from Arabidopsis shoots using a continuous 0 - 4.4% (w/v) PVP/28% colloidal density gradient. The dark green band is the thylakoids and other contaminations as indicated in the top of the gradient solutions. Mitochondrial fraction appeared as a white-greenish band closer to the bottom of the tube. [Please click here to view a larger version of this figure.](#)

Figure 3: Separation of mitochondrial proteins by SDS-PAGE and immunodetection using specific antibodies. (A) Mitochondria isolated from two-week-old water-cultured *Arabidopsis thaliana* wild type (Columbia-0, Col-0) plants were subjected to SDS-PAGE and probed with antibodies raised against NADH:ubiquinone oxidoreductase subunit S4 (Ndufs4, At5g67590). Molecular-weight unstained markers were loaded on the outer lane of the gel and the size of ten representative bands are indicated in kilo Daltons (kDa). The apparent molecular mass of the Ndufs4 protein detected is 18 kDa. Protein abundance is shown relative to the value of 2 μ g total protein. (B) Two-week-old seedlings grown on Gamborg's B5 media with 3% (w/v) sucrose and 0.8% agar (w/v) were exposed to 750 μ E m⁻² s⁻¹ highlight (HL) and harvested after 6 h. Mitochondria were purified and the mitochondrial proteins were separated by SDS-PAGE and probed with antibodies raised against alternative oxidase (AOX). The presence of an immunodetectable AOX protein with an apparent molecular mass of 34 kDa is indicated. Protein abundance is shown relative to the value of control (2 μ g). [Please click here to view a larger version of this figure.](#)

Figure 4: Representative traces of O₂ consumption by isolated plant mitochondria. (A) Mitochondria isolated from *Arabidopsis thaliana* wild-type plants (Col-0) as outlined above were analyzed for outer membrane integrity prior to oxygen consumption measurements. 30 μ L of isolated mitochondria (150 μ g total mitochondrial protein) were used. Mitochondrial integrity was determined as 90% as described above. (B) Oxygen consumption measurements were performed using 30 μ L of isolated mitochondria (150 μ g total mitochondrial protein) from *Arabidopsis thaliana* wild-type plants. Total mitochondrial respiration and the AOX pathway were determined. From these data, oxygen consumption through the cytochrome c pathway and AOX pathway can be determined. [Please click here to view a larger version of this figure.](#)

Grinding medium	
Chemical	Concentration
Sucrose	0.3 M
tetrasodium pyrophosphate (Na ₄ P ₂ O ₇ · 10 H ₂ O)	25 mM
EDTA disodium salt	2 mM
Potassium phosphate monobasic (KH ₂ PO ₄)	10 mM
Polyvinylpyrrolidone (PVP40)	1% (w/v)
Bovine serum albumin (BSA)	1% (w/v)
Deionized water	
Adjust pH to 7.5 (using HCl)	
NOTE: For 300 mL grinding medium, 1.06 g sodium ascorbate (final concentration: 17.84 mM) and 0.74 g cysteine (final concentration: 20.36 mM) are added just prior to use. Check pH after addition and adjust to 7.5 with 1 M NaOH if required.	
2X wash buffer	
Chemical	Concentration
Sucrose	0.6 M
TES	20 mM
Bovine serum albumin (BSA)	0.2% (w/v)
Deionized water	
Adjust pH to 7.5 (using NaOH)	
Gradients	
Heavy Gradient solution (4.4% (w/v) PVP)	2 gradient tubes
2X wash buffer	17.5 mL
Colloidal density gradient	9.8 mL
PVP-40 (20% (w/v))	7.7 mL
Light Gradient Solution (0% (w/v) PVP)	2 gradient tubes
2X wash buffer	17.5 mL
Colloidal density gradient	9.8 mL
Deionized water	7.7 mL

Table 1: Composition of buffers and gradients used for mitochondria isolation.

	Abbreviation	Concentration of stock solutions	Storage	Final concentration	Volume added for 1 ml reaction
Substrates					
Cytochrome c	Cyt c	2.5 mM (in H ₂ O)	-20 °C	25 µM	10 µl
NADH	NADH	0.1 M (in H ₂ O)	-20 °C	1 mM	10 µl
Succinate	Succ	500 mM (in H ₂ O)	-20 °C	5 mM	10 µl
Inhibitors					
Antimycin A	AA	1 mM (in EtOH)	-20 °C	5 µM	5 µl
Cyanide	KCN	100 mM (in H ₂ O)	4 °C	1 mM	10 µl
Myxothiazol	Myxo	500 µM (in EtOH)	-20 °C	2.5 µM	5 µl
<i>n</i> -Propyl gallate	nPG	100 mM (in EtOH)	-20 °C	500 µM	5 µl
Effectors					
ADP	ADP	100 mM (in H ₂ O)	-20 °C	1 mM	10 µl
Ascorbate	Asc	500 mM (in H ₂ O)	Make fresh on the day of use	10 mM	20 µl
ATP	ATP	100 mM (in H ₂ O)	-20 °C	500 µM	5 µl
Dithiothreitol	DTT	1 M (in H ₂ O)	Make fresh on the day of use	10 mM	10 µl
Pyruvate	Pyr	1 M (in H ₂ O)	-20 °C	10 mM	10 µl
Detergent		10% (v/v) (in H ₂ O)	4 °C	0.05% (v/v)	5 µl

Table 2: List of substrates, inhibitors and effectors used for oxygen consumption measurements.

Discussion

Typically, isolation of mitochondria from *Arabidopsis* leaves yields up to 3 mg of mitochondria from approximately 80 - 100 3 - 4-week old plants, although yields of greater than 5 mg can often be achieved with thorough grinding. The yield varies with growth conditions and decreases dramatically as leaves senesce, although mitochondria structure seems to be well maintained during senescence⁹. One of the most critical features to obtain a good yield is the method of grinding to lyse cells to release mitochondria. While a number of mechanical grinding apparatus are available for purchase, for *Arabidopsis* grinding in a mortar and pestle achieves consistently good results in terms of yield, as it lyses the cells with little damage to organelles. While mechanical grinders are fast, they require optimization and the amount of grinding required can vary according to the tissue. With a mortar and pestle, it is often convenient and more efficient if the tissue is sliced or cut with a knife or scissors before grinding. As outlined, all steps need to be carried out at 4 °C and the whole procedure from the end of grinding to obtaining a washed pellet of purified mitochondria should take approximately 4 h. As traces of detergents or other reagents on tubes or gradient pourers can dramatically reduce yield, all components used in these procedures are washed without detergent and not used in other procedures. Finally, it is important that the mitochondria are sufficiently separated from the other fractions on the gradient to allow them to be removed and washed. If they are too near the bottom of the tube, it means that the mixing of the light and heavy solutions needs to be adjusted, to allow more of the heavy solution to pour before mixing. Conversely, if the band appears diffuse and is high in the tube, mixing needs to occur a little earlier.

The method outlined here can be readily used for isolation of mitochondria from *Arabidopsis* flower and root tissue^{11,12}. For roots, it is convenient to grow in hydroponic culture and need 100 g of fresh weight to obtain 2 mg amounts of mitochondria. For grinding roots should be cut into small pieces prior to grinding in a mortar and pestle, and increase yields can be obtained by re-grinding the root tissue, either in a mortar and pestle or in a blender. For floral tissue where mitochondrial function is often enhanced¹³, grinding with a mortar and pestle works well, but the limited amount of material means that a large amount of plants needs to be grown to harvest tissue. Mitochondria have been isolated from a variety of *Arabidopsis* organs using similar methods or slight modifications^{14,15} and from rice (*Oryza sativa*)¹⁶.

The limitations of the method described above are i) the large amounts of seeds required for the mitochondrial isolation, ii) only specific tissues from *Arabidopsis* and rice applied in this isolation method, iii) small quantities of various contaminants (such as peroxisomal proteins) still existed in the purified mitochondria. Mitochondria obtained using the methods described above are suitable for a variety of studies, ranging from oxygen uptake studies to quantitative mass spectrometry analyses of protein abundance. Gradient purified mitochondria will still contain small quantities of various contaminants, such as peroxisomal proteins³. A comparison with defined organelle lists can be used to determine changes in mitochondrial proteins, as long as the amount of contamination by non-mitochondrial proteins is small (<10%) and similar samples are compared, so the type and degree of non-mitochondrial proteins is similar. Analyses of protein abundance by SDS-PAGE or other gel-based approaches can be carried out with relatively small amounts of mitochondria (2 - 20 µg), using varying amounts of mitochondria to check the linearity of detection by antibodies (**Figure 3**). While porin (voltage dependent anion channel (VDAC)) is often used as a loading control for quantification, in our experience the relatively large abundance of this protein can mean that the response is often not linear if large amounts of protein are loaded on the gel, so linearity of antibody response should always be checked when using such loading controls. The significance of this approach of isolating mitochondria is that unlike gradients that use sucrose as the material to form the density gradient, colloidal density gradients do not require osmotic re-adjustment of the purified mitochondria as is required with sucrose. This means that there is less chance of

rupturing the purified mitochondria and after washing to remove the colloidal density material, they can be directly used in a variety of assays or applications.

Tissue blots, where mitochondrial proteins are detected from whole leaf or tissue extracts, are an attractive approach to measure the amount of mitochondrial proteins in that tissue. Given the generally low volume of the mitochondria compared to other organelles, the detection of mitochondrial protein on whole tissue extracts needs to be interpreted with some caution, as mitochondrial protein may be beyond detection in such approaches. Careful controls, where purified mitochondria are electrophoresed along with tissue extracts to ensure identical migration and linearity of detection, need to be carried out to have confidence in such approaches.

With the help of a Clark-type oxygen electrode, changes in the oxygen partial pressure of a solution can be measured. The actual electrode consists of a platinum cathode and a silver anode, which are connected by a KCl bridge and covered by an electrolyte-moistened paper (cigarette paper) and an oxygen-permeable membrane (polytetrafluoroethylene membrane). A voltage of 600 - 700 mV leads to the reduction of oxygen, giving a linear relationship between oxygen concentration and voltage. Oxygen electrodes are available commercially from different companies. Each company will have its own instructions regarding the assembly and setup of the oxygen electrode. However, in general the silver anode and platinum cathode of the electrode disk need to be cleaned with the help of an electrode cleaning kit or an eraser pen before assembly. It is important to ensure that air bubbles do not form during assembly (e.g. between the polytetrafluoroethylene diaphragm and the cigarette paper). After assembly, the electrode disk needs to be attached to the chamber with the platinum cathode facing upwards at the base of the reaction chamber. The chamber itself is surrounded by a water jacket ensuring temperature control. It is extremely important that the chamber is always left full of water, since the membrane will dry out and crack otherwise. Likewise, the membrane must not be touched by pipettes or syringes when adding compounds to the chamber, to avoid tearing.

Prior to the assay, the respiration medium should be warmed to the same temperature as the assay chamber (in most cases 25 °C) and the membrane should be allowed to equilibrate in respiration buffer for a few min. After closing the plunger for oxygen consumption assays, the addition of effector molecules should be carried out using either non-disposable microliter syringes (e.g. micro syringes) or disposable gel loading pipette tips. If using micro syringes, it has to be ensured that the syringe is thoroughly rinsed with 100% ethanol and water between additions, to avoid contaminating stock solutions. This also applies for the washing of the chamber between measurements. Most reagents used in oxygen consumption assays are soluble in water and can easily be removed by multiple rinsing with water (approximately five times) between measurements. However, some chemicals used for assays are only soluble in organic solvents, such as antimycin A, myxothiazol, and nPG. Therefore, the chamber needs to be rinsed with an organic solvent (50% (v/v) ethanol) between assays to remove residual traces of these molecules, and then with water (approximately five times) to deplete residues.

Disclosures

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