CHAPTER 13

Isolation of Mitochondria from Cells and Tissues

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Mitochondria are complex organelles at the center of cellular metabolism, apoptosis, and signaling. They continue to be the subject of intense basic investigation to understand their composition and function, but they have also captivated the attention of clinical researchers because of the growing knowledge of the (sometimes unexpected) roles of mitochondria in human diseases and aging. A full understanding of these intriguing organelles often requires their purification from cells or tissues under specific physiological or pathological conditions. Here we provide some introductory considerations for those interested in purifying mitochondria for subsequent downstream biophysical, structural, and functional analysis.

TIPS FOR ISOLATING MITOCHONDRIA FROM CELLS AND TISSUES

Although mitochondria can be isolated from a variety of tissues and cells, there is no universal procedure that works equally well for all of them. Every isolation method follows a basic scheme: preparation of a homogenate followed by differential centrifugation and, if necessary, density-gradient centrifugation. However, the unique characteristics of the cells or tissue and the experimental purpose for which the mitochondria are isolated determine the details, such as choice of homogenizer, buffer composition, and acceptable levels of contaminating organelles. Therefore, although not technically demanding, successful isolation of mitochondria requires an understanding of the nature of the cells or tissue being used and may require that some pilot experiments be performed. The following points should be applicable to most procedures.

- Perform all steps at 0°C –4°C.
- Work quickly and purify the mitochondria only to the degree necessary for your purposes. Each manipulation will result in losses.
- Keep the cell and organelle suspensions dilute during the procedure to reduce the potential for trapping and agglutination.
- Several small preparations give better yields than a single large one. Scaling up does not translate into a proportional increase in yield.
- The solutions used should maintain the integrity of the mitochondria, facilitate the fractionation, and be compatible with subsequent procedures. Typically, mitochondria are isolated in a solution containing a buffer to maintain the pH of the homogenate, a chelating agent, and a sugar to

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maintain the tonicity of the organelles. Isotonic (0.25 M) sucrose has been used almost exclusively for cell fractionation, but mannitol has advantages over sucrose for preparing mitochondria. The source from which the mitochondria is being isolated will dictate the exact composition of the buffer solution.

- Preparation of the homogenate is a critical step in the isolation of mitochondria. The goal is to break open the cells without damaging the subcellular organelles. This is the step to examine first if you have problems.
- The mitochondria will be contaminated with other organelles. The relative purity of the mitochondrial preparation can be determined by assaying for marker proteins or enzymes for mitochondria and the other organelles that are likely to be present. The levels of these markers should be considered relative to their levels in the whole homogenate. The following are frequently used marker enzymes, but western blotting of compartment-specific proteins can also be used: mitochondria—cytochrome *c* oxidase, succinate dehydrogenase; lysosomes—acid phosphatase, β-galactosidase; and peroxisomes—catalase, uricase.
- The mitochondrial fraction can be examined with an electron microscope, although the results are not as reliable an indicator of yield and purity as are enzyme assays and western blots.

PROTOCOLS FOR THE ISOLATION OF MITOCHONDRIA

Three accompanying protocols can be used to isolate and enrich for mitochondria. The first two, Protocol 1: Isolation of Mitochondria from Tissue Culture Cells and Protocol 2: Isolation of Mitochondria from Animal Tissue, can be used with tissue culture cells and tissues such as rodent liver, respectively (Clayton and Shadel 2014a,b). The technical problems encountered when isolating mitochondria from these two sources are sufficiently different through the washed mitochondrial pellet step that separate protocols are warranted. This washed pellet is suitable for many experimental purposes, or it can be purified further by density gradient centrifugation (see Protocol 3: Purification of Mitochondria by Sucrose Step Density Gradient Centrifugation [Clayton and Shadel 2014c]).

REFERENCES

Clayton DA, Shadel GS. 2014a. Isolation of mitochondria from tissue culture cells. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot080002.

Clayton DA, Shadel GS. 2014b. Isolation of mitochondria from animal tissue. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot080010.

Clayton DA, Shadel GS. 2014c. Purification of mitochondria by sucrose step density gradient centrifugation. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot080028.

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Protocol 1

Isolation of Mitochondria from Tissue Culture Cells

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The number of mitochondria per cell varies substantially from cell line to cell line. For example, human HeLa cells contain at least twice as many mitochondria as smaller mouse L cells. This protocol starts with a washed cell pellet of 1–2 mL derived from ~10^9 cells grown in culture. The cells are swollen in a hypotonic buffer and ruptured with a Dounce or Potter–Elvehjem homogenizer using a tight-fitting pestle, and mitochondria are isolated by differential centrifugation.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Cell pellet derived from 1–5 × 10^9 tissue culture cells

MS homogenization buffer (1× and 2.5×) <R>

MS homogenization buffer is an iso-osmotic buffer used to maintain the tonicity of the organelles and prevent agglutination.

RSB hypo buffer <R>

RSB is a hypotonic buffer used for swelling tissue culture cells.

Equipment

Centrifuge tubes

Dounce homogenizer (15 mL) with a tight-fitting B pestle or Potter–Elvehjem homogenizer (5 mL) with a Teflon pestle (see Steps 1 and 3)

Phase contrast microscope

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METHOD

The solutions, tubes, and homogenizer should be prechilled on ice. All centrifugation steps are at 4°C.

1. Resuspend the cell pellet in 11 mL of ice-cold RSB hypo buffer and transfer the cells to a 15-mL Dounce homogenizer.

   Alternatively, as described by Frezza et al. (2007), resuspend the cell pellet in 9 mL of ice-cold RSB hypo buffer and transfer 3 mL of the cells at a time to a 5-mL Potter–Elvehjem homogenizer with a Teflon pestle.

2. Allow the cells to swell for 5–10 min. Check the progress of the swelling using a phase-contrast microscope.

3. Break open the swollen cells with several strokes of the B pestle. For each stroke, press the pestle straight down the tube, maintaining a firm, steady pressure.

   If a Potter–Elvehjem homogenizer is used in Step 1, then break open the cells with the Teflon pestle rotating at ~1600 rpm.

4. Check the degree of homogenization with a phase-contrast microscope.

   Naked nuclei (smooth spheres with obvious nucleoli inside), smaller organelles (dark, granular objects), and a small number of unbroken cells (large spheres with a granular appearance) should be present if cell lysis was successful. Eight to nine naked nuclei for every whole cell is a very good result. Trying for anything better usually results in increasing the number of damaged nuclei, which increases the number of mitochondria trapped in the nuclear pellet during the first centrifugation.

   See Troubleshooting.

5. Immediately add 8 mL of 2.5× MS homogenization buffer to give a final concentration of 1× MS homogenization buffer. Cover the top of the homogenizer with Parafilm and mix by inverting a couple of times. (Save a portion of the homogenate if marker enzyme assays are to be performed later.)

6. Transfer the homogenate to a centrifuge tube for differential centrifugation. Rinse the homogenizer with a small amount of 1× MS homogenization buffer and add it to the homogenate. Bring the volume to 30 mL with 1× homogenization MS buffer.

7. Centrifuge the homogenate at 1300*g* for 5 min to remove nuclei, unbroken cells, and large membrane fragments.

8. Pour the supernatant into a clean centrifuge tube.

   The top of the pellet will be loose, so be careful not to collect it with the supernatant.

9. Repeat Steps 6 and 7 two more times.

10. Transfer the supernatant to a clean centrifuge tube and pellet the mitochondria at 7,000*g*–17,000*g* for 15 min.

11. Discard the supernatant and wipe out the inside of the tube with a Kimwipe.

12. Wash the mitochondria by resuspending the pellet in 1× MS buffer and repeating the 7,000*g*–17,000*g* sedimentation.

    This wash is not necessary if a density gradient will be performed (see Protocol 3: Purification of Mitochondria by Sucrose Step Density Gradient Centrifugation [Clayton and Shadel 2014]).

13. Discard the supernatant and resuspend the pellet in a buffer suitable for subsequent work.

    The mitochondria can be stored at −80°C for at least 1 yr for some purposes (e.g., protein isolation).

TROUBLESHOOTING

Problem (Step 4): Too many or not enough cells have lysed.

Solution: Homogenization works best if the cells are resuspended in at least 5–10× the volume of the cell pellet and if the cell suspension occupies at least half the volume of the homogenizer. Homogenization should be performed as quickly as possible because it is performed in a hypotonic buffer. The

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Dounce homogenizer disrupts swollen tissue culture cells by pressure change. As the pestle is pressed down, pressure around the cell increases. When the cell slips past the end of the pestle, the sudden decrease in pressure causes the cell to rupture. If the pestle is very tight fitting, there may be some mechanical breakage as well. If an excessive number of strokes are needed for good cell breakage, a tighter-fitting homogenizer is needed.

DISCUSSION

This basic protocol can be modified to suit special purposes. For example, if the mitochondria are being purified to isolate mitochondrial DNA, contamination with nuclei, not the small organelles, is a problem and the following modifications could be made: Harvest the cells in stationary growth phase when the fewest cells will be actively dividing, substitute $CaCl_2$ for $MgCl_2$ in the RSB hypo buffer to stabilize the nuclear membrane, omit washing the mitochondrial pellet, omit any density gradient purification, resuspend and lyse the mitochondrial pellet from Step 10, and purify the mitochondrial DNA from any remaining nuclear DNA (Hudson et al. 1968).

RECIPES

MS Homogenization Buffer (1×)

210 mM mannitol
70 mM sucrose
5 mM Tris-HCl (pH 7.5)
1 mM EDTA (pH 7.5)

The buffer should be ice cold before use.

MS Homogenization Buffer (2.5×)

525 mM mannitol
175 mM sucrose
12.5 mM Tris-HCl (pH 7.5)
2.5 mM EDTA (pH 7.5)

The buffer should be ice cold before use.

RSB Hypo Buffer

10 mM NaCl
1.5 mM $MgCl_2$
10 mM Tris-HCl (pH 7.5)

The buffer should be ice cold before use.

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Clayton DA, Shadel GS. 2014. Purification of mitochondria by sucrose step density gradient centrifugation. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot080028.

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Hudson B, Clayton DA, Vinograd J. 1968. Complex mitochondrial DNA. *Cold Spring Harbor Symp Quant Biol* **33:** 435–442.