Chapter 10

Oxidative Phosphorylation: Synthesis of Mitochondrially Encoded Proteins and Assembly of Individual Structural Subunits into Functional Holoenzyme Complexes

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Abstract

The bulk of ATP consumed by various cellular processes in higher eukaryotes is normally produced by five multimeric protein complexes (I–V) embedded within the inner mitochondrial membrane, in a process known as oxidative phosphorylation (OXPHOS). Maintenance of energy homeostasis under most physiological conditions is therefore contingent upon the ability of OXPHOS to meet cellular changes in bioenergetic demand, with a chronic failure to do so being a frequent cause of human disease. With the exception of Complex II, the structural subunits of OXPHOS complexes are encoded by both the nuclear and the mitochondrial genomes. The physical separation of the two genomes necessitates that the expression of the 13 mitochondrially encoded polypeptides be co-ordinated with that of relevant nuclear-encoded partners in order to assemble functional holoenzyme complexes. Complex biogenesis is a highly ordered process, and several nuclear-encoded factors that function at distinct stages in the assembly of individual OXPHOS complexes have been identified.

Key words: Mitochondria, oxidative phosphorylation mtDNA, pulse-chase labeling, mitochondrial translation, holoenzyme assembly, blue native PAGE.

1. Introduction

The mitochondrial content of most eukaryotic cells is largely a reflection of their bioenergetic requirements. Transient increases in cellular ATP demand can generally be met by the existing population of mitochondria, because the abundance of the five multimeric protein complexes (I–V) that catalyze oxidative phosphorylation (OXPHOS) is in slight excess of that required to maintain energy homeostasis under normal conditions *(1)*; however, chronic

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bioenergetic shortfalls resulting from either genetic perturbations or environmental and physiological stimuli trigger an adaptive response, in which the mitochondrial content of the cell is increased in an attempt to restore energy homeostasis *(2, 3)*.

Both the maintenance of existing levels of OXPHOS complexes and the adaptive increases in their abundance that accompany mitochondrial biogenesis require the de novo synthesis of individual structural subunits. Thirteen proteins critical to the biogenesis of Complexes I, III, IV, and V are encoded by mitochondrial DNA (mtDNA), and a large number of nuclear-encoded accessory factors regulate their expression at either the transcriptional or the translational level *(4)*. The stability of newly synthesized mitochondrial proteins is dependent upon their insertion into the inner mitochondrial membrane and subsequent assembly with relevant nuclear partners to form functional holoenzyme complexes, a process that is frequently facilitated by one or more nuclear-encoded assembly factors. The apparent interdependence of the biogenesis of some OXPHOS complexes may be explained by their eventual organization into higher order structures termed supercomplexes *(5)*.

Changes in both the synthesis and the stability of mitochondrially encoded proteins can be readily quantified in cultured cells by labeling with radioactive (^{35}S) methionine in the presence of the appropriate inhibitors of cytoplasmic translation. Differences in the absolute levels of the holoenzymes themselves can be assessed by blue-native polyacrylamide gel electrophoresis (BN-PAGE), followed by conventional immunoblotting with commercially available antibodies. Both of these techniques are straightforward and may provide considerable mechanistic insight into the molecular genetic basis of not only human disease but also physiological adaptation to a range of intrinsic and extrinsic stimuli.

2. Materials

2.1. Pulse-Chase Labeling of the Mitochondrial Translation Products

2.1.1. Labeling of Mitochondrially Synthesized Proteins with Radioactive (^{35}S) Methionine and Cysteine

1. Labeling medium: Dulbecco's Modified Eagle's Medium (DMEM) without methionine and cysteine (Gibco – Invitrogen Corp., Carlsbad, CA), supplemented with 10% dialyzed fetal bovine serum (FBS), 1X glutamax and 110 mg/l sodium pyruvate (*see* **Note 1**). Store at 4°C.
2. Regular DMEM supplemented with 10% FBS. Store at 4°C.
3. Phosphate-buffered saline (PBS) reconstituted from tablets and sterilized by autoclaving. Store at room temperature.
4. Inhibitors of cytoplasmic translation: emetine (Sigma–Aldrich, St. Louis, MO) for pulse-labeling or anisomycin (Sigma–Aldrich)

for chase-labeling. In each case, prepare a 2 mg/ml solution in PBS, and sterilize by passing through a 0.2-μm syringe filter (Sarstedt, Newton, NC). Make fresh as required.

5. Pro-Mix L-(^{35}S) in vitro cell-labeling mix, >1,000 Ci/mmol (EasyTag, PerkinElmer Life and Analytical Sciences, Woodbridge, ON) (*see* **Note 2**). Store at 4°C. Observe handling and storage conditions required for this particular radioactive isotope.
6. Chloramphenicol (CAP, Sigma–Aldrich) for chase-labeling. Prepare a 1 mg/ml solution in regular DMEM without serum (*see* **Note 3**), then sterilize by passing through a 0.2-μm syringe filter. Stable at 4°C for up to 1 week.
7. Cell lifters (Corning, Inc. Life Sciences, Lowell, MA).

2.1.2. Sample Preparation

1. Gel loading buffer (2X): 186 mM Tris–HCl, pH 6.7 – 6.8, 15% glycerol, 2% sodium dodecyl sulfate (SDS), 0.5 mg/ml bromophenol blue, 6% β-mercaptoethanol (β-ME). Store at room temperature. Add β-ME just before use.
2. Micro-BCA^{TM} Protein Assay Kit (Pierce Biotechnology, Rockford, IL).
3. High Intensity Ultrasonic Processor (Sonics & Materials, Inc., Danbury, CT).

2.1.3. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Separating buffer (4X): 1.5 M Tris–HCl (pH 8.8), 8 mM EDTA-Na_2, 0.4% SDS. Store at room temperature.
2. Stacking buffer (4X): 0.5 M Tris–HCl (pH 6.8), 8 mM EDTA-Na_2, 0.4% SDS. Store at room temperature.
3. Thirty percent acrylamide/bisacrylamide solution (37.5:1) (Bioshop Canada, Burlington, ON, Canada). Avoid exposure to unpolymerized solution, as it is a neurotoxin. Store at 4°C.
4. *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED, Bioshop, Canada). Store at 4°C.
5. Ammonium persulfate (APS): prepare a 10% solution in double-distilled water. Make fresh as required.
6. Running buffer (1X): To 3 l double-distilled water (total volume required for one run), add 9.08 g Tris base, 43.25 g glycine, and 3.0 g SDS. Do not pH. Store at room temperature.
7. Molecular weight markers: Page Ruler^{TM} Pre-stained Protein Ladder (Fermentas, Glenburnie, MD).
8. WIZ Peristaltic Pump (Teledyne Isco, Lincoln, NE).

2.1.4. Generation and Analysis of the Data

1. SGD2000 Digital Slab Gel Dryer (Thermo Fisher Scientific, Waltham, MA).

2. Storm 840 Gel and Blot Imaging System (GE Healthcare).

2.2. Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE)

2.2.1. Cell Culture and Sample Preparation

1. PBS and DMEM supplemented with 10% FBS, prepared and stored as described in **Section 2.1.1.**
2. Trypsin solution (0.05% final in PBS) (Gibco – Invitrogen Corp.). Store at 4°C.
3. Bovine serum albumin (BSA) diluted to 1 mg/ml in double-distilled water and Bradford reagent (Bio-Rad, Hercules, CA).
4. Digitonin (EM Biosciences, San Diego, CA) resuspended at 4 mg/ml in PBS (*see* **Note 4**).
5. Blue native (BN) sample buffer: 0.5 ml 3X gel buffer (1.5 M aminocaproic acid [Sigma–Aldrich], 150 mM Bis-tris [Bioshop, Canada], pH 7.0), 0.5 ml 2 M aminocaproic acid, and 4 µl 500 mM EDTA (*see* **Note 5**). Stable for 6–12 months when stored at 4°C.
6. Lauryl maltoside (Roche) as a 10% solution in double-distilled water (*see* **Note 6**).
7. Coomassie Brilliant Blue G-250 (SBG) (Bio-Rad) as a 5% solution in 0.75 mM aminocaproic acid (*see* **Note 7**). Store indefinitely at 4°C.

2.2.2. BN-PAGE

1. Acrylamide/bisacrylamide (AB) mix: 48% acrylamide, 1.5% bisacrylamide [99.5%T, 3%C] (Bioshop, Canada) (*see* **Note 8**). Store for 6–12 months at 4°C.
2. 3X Gel buffer (*see* **Section 2.2.1**).
3. 87% Glycerol (EM Biosciences) stock solution (in water). Store indefinitely at room temperature.
4. Colorless cathode buffer: 15 mM Bis-tris, 50 mM Tricine (EM Biosciences), pH 7.0.
5. Blue cathode buffer: colorless cathode buffer containing 0.02% SBG.
6. Anode buffer: 50 mM Bis-tris, pH 7.0 (*see* **Note 9**).
7. APS and TEMED, prepared and stored as described in **Section 2.1.3**.
8. Protein standards: high molecular weight native marker kit (Pharmacia) (*see* **Note 10**).

2.2.3. Western Blotting and Immunodetection

1. Trans-blot SD semi-dry transfer cell (Bio-Rad).
2. Transfer buffer: To 1 l double-distilled water, add 5.8 g Tris base, 2.93 g glycine, 0.75 g SDS, and 200 ml methanol. Do not pH. Store at room temperature.

3. Nitrocellulose (Pall Corporation, Mississauga, ON, Canada) and Whatman 3 M paper (Schleicher & Schuell, New Jersey, NJ).
4. Tris-buffered saline (TBS) (10X): To 2 l of double-distilled water, add 48.4 g Tris base, 160 g NaCl. Do not pH (*see* **Note 11**). Store indefinitely at room temperature.
5. Barnstead Lab Line Maxi Rotator (VWR scientific, Mississauga, ON, Canada).
6. Blocking solution: 5% BSA dissolved in 1X TBS supplemented with 0.1% Tween-20 (TBS-T).
7. Primary and secondary antibody solution: TBS-T supplemented with 2% BSA.
8. Primary antibodies (Mitosciences, Eugene, OR) for Complexes I (anti-39 kDa), II (anti-SDHA), III (anti-core 1), IV (anti-COX I or anti-COX IV), and V (anti-ATPase α).
9. Secondary antibody: anti-mouse IgG conjugated to horseradish peroxidase (Cedarlane Laboratories, Burlington, ON, Canada). Enhanced chemiluminescent (ECL) reagents (Cell Signaling Technology, Danvers, MA) and Hyclone CL film (Denville Scientific, Inc., Metuchen, NJ).
10. Stripping solution: To 0.5 l of double-distilled water, add 31.5 ml Tris–HCl (pH 7.5), 10 g SDS, and 3.9 ml β-ME (*see* **Note 12**).
11. Sciera shaking water bath (Bellco Biotechnology, Vineland, NJ).

3. Methods

Pulse-labeling of mitochondrial translation products allows for an assessment of both the expression of individual proteins and the global rate of mitochondrial protein synthesis. Chase-labeling further permits for an evaluation of the stability of mitochondrially encoded proteins, and by extension, their assembly into the multimeric holoenzyme complexes of OXPHOS. In both cases, cells are exposed to a mixture of radiolabeled methionine and cysteine in the presence of an inhibitor of cytoplasmic translation, which allows for the specific radiolabeling of mitochondrially encoded proteins. There are, however, three main differences between pulse- and chase-labeling: first, the length of the chase, defined as the incubation time in regular, "cold" medium following removal of the radiolabel; second, the type of inhibition of cytoplasmic translation (i.e., irreversible versus reversible); and third,

exposure to chloramphenicol, a reversible inhibitor of mitochondrial translation that is only used for chase-labeling. With pulse-labeling, the short duration of the chase (i.e., 10 min) allows for the use of an irreversible inhibitor of cytoplasmic translation such as emetine. In contrast, a reversible inhibitor of cytoplasmic translation is required when chase-labeling, because cells are maintained in culture for a period of time that is sufficient (up to 17 h) to quantify the rate of degradation of radiolabeled proteins. For this purpose, we use anisomycin, although several other groups prefer cycloheximide *(6, 7)*. Finally, in chase-labeling, cells are exposed to chloramphenicol prior to incubation with the radioisotope. This results in the accumulation of a pool of nuclear-encoded structural subunits within the mitochondria, an event which facilitates the assembly of nascent OXPHOS complexes subsequent to the radiolabeling of mitochondrially encoded subunits *(8)*.

As outlined in this chapter, the pulse-chase labeling procedure can be applied to all types of adherent cells, independent of either the species of origin or their proliferative state (i.e., dividing versus terminally differentiated) and regardless of whether they are transformed, primary, or immortalized. It is important to recognize, however, that the characteristic pattern of mitochondrial translation is unique to each individual species, even when the identical cell type is being considered. Variation across both the individuals and the tissues within a single species is also possible. This variation can be qualitative, with differences in the electrophoretic mobility of a specific protein *(6, 7)* or quantitative, with differences in the overall abundance of mitochondrial translation products (also *see* **Fig. 10.1**). Such qualitative differences can be due to neutral polymorphisms, while quantitative differences likely reflect different energetic requirements across cell types. It is therefore essential that all of the appropriate controls be included in each experiment.

Pioneered in the early 1990s by Schagger and colleagues *(9, 10)*, BN-PAGE has emerged as the technique of choice to examine the assembly and abundance of OXPHOS complexes within the inner mitochondrial membrane. In its original form, Coomassie dyes were used to impart the charge shift necessary for detergent solubilized proteins to be fractionated by size in a non-ionic gel and buffer system. The technique has since been refined to permit studies of the organization of OXPHOS complexes into higher order structures known as supercomplexes *(11)*; however, it is described here in its simplest form, which results in the release of OXPHOS complexes from the inner mitochondrial membrane in either their monomeric (Complexes I, II, IV, and V) or dimeric forms (Complex III). Detailed instructions are provided for using digitonin to prepare an enriched mitoplast fraction starting from whole cells *(12)*, an approach we favor because much smaller amounts of starting material are required for downstream analyses.

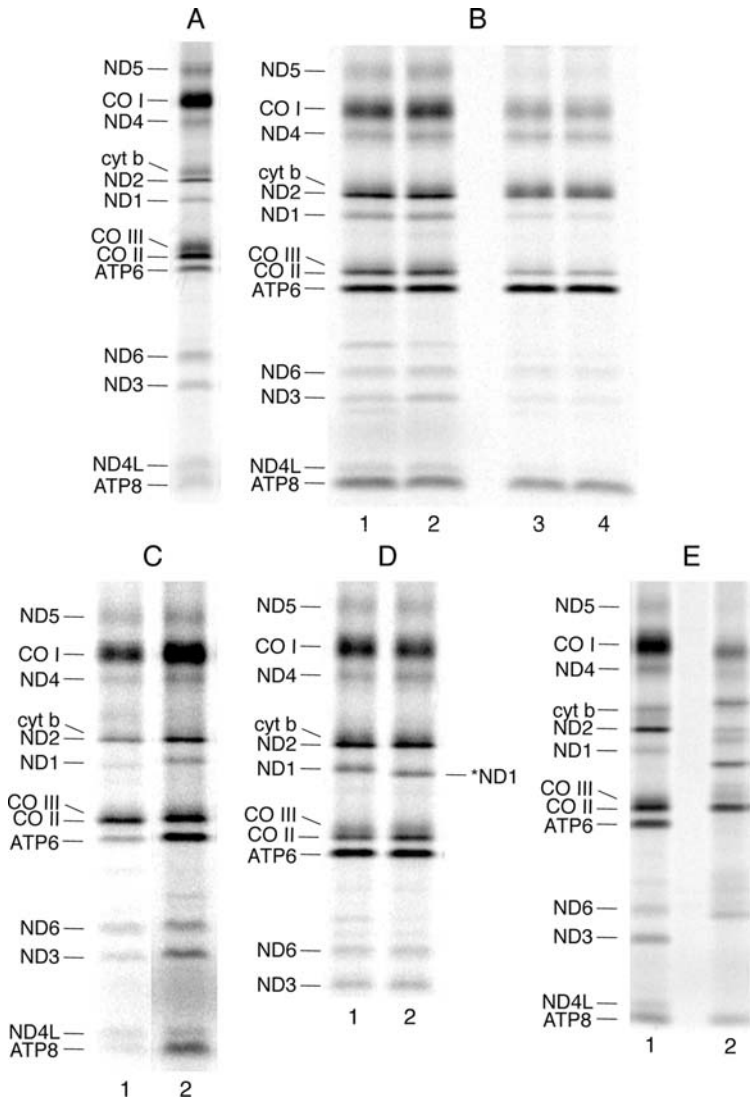

Fig. 10.1. *In vivo analysis of mitochondrial translation by pulse-chase labeling. Panel A*: Typical pattern of pulse-labeled mitochondrial translation products in human cultured cells, shown here for immortalized myoblasts. The 13 mitochondrially synthesized proteins are indicated at the left of the panel: ND, subunits of Complex I; CO, subunits of Complex IV; ATP, subunits of Complex V; and cyt b, subunit of Complex III. *Panel B*: Pulse- (1,2) and chase- (3,4) labeling of two lines of immortalized human fibroblasts. Note that in chase-labeling the two Complex V subunits are preferentially stabilized, a characteristic event resulting from the addition of CAP. *Panel C*: Increased levels of mitochondrial translation products in the transformed cell line HEK293 (2), when compared to a line of immortalized myotubes (1). The two lanes are part of the same gel and have been placed side-by-side to facilitate comparison. *Panel D*: Different migration of the ND1 subunit (most likely due to a neutral polymorphism) in two different lines of immortalized human fibroblasts analyzed by pulse-labeling. *Panel E*: Difference between human and mouse cultured cells in the overall pattern of pulse-labeled mitochondrial translation products, shown here for the human osteosarcoma line 143B (1) and the mouse myeloma line A9 (2). In all panels, each lane contains 50 μg of total cellular protein.

If the starting material is either abundant or prevents the use of such an approach (e.g., autopsy/biopsy material), mitochondria may be isolated by differential centrifugation prior to solubilization in lauryl maltoside. It is also important to note that although we focus on the analysis of cultured human cell lines, BN-PAGE can also be used to study OXPHOS complexes in a range of tissues and cell types derived from both model organisms and non-traditional species. The primary limitation when extending this methodology to investigate non-traditional paradigms, however, is the availability of primary antibodies that will recognize structural subunits of the various OXPHOS complexes. While some commercially available antisera against human proteins do crossreact with their homologues in other species *(13)*, these must be tested empirically.

3.1. Pulse-Chase Labeling of the Mitochondrial Translation Products

3.1.1. Labeling of Mitochondrially Synthesized Proteins with Radioactive (^{35}S) Methionine and Cysteine

1. One 60-mm tissue culture plate is required for each cell line to be labeled. Split dividing cells such that on the day of the experiment, they are between 75 and 90% confluent (*see* **Note 13**).
2. If cells will be chase-labeled, prepare the CAP solution.
3. For chase-labeling only, aspirate growth medium from each plate 22–24 h prior to the start of the labeling procedure, and add 4.8 ml fresh growth medium and 200 µl CAP solution (total volume of 5 ml/plate, final CAP concentration of 40 µg/ml).
4. At least 30 min before the start of the labeling procedure, pipette the total volumes of labeling medium (2 ml/plate) and of DMEM+10% FBS (5 ml/plate) that are required for the entire experiment into two separate tissue culture plates, and place them in the incubator. This step will allow the media to equilibrate to 5% CO_2 and 37°C (*see* **Note 14**).
5. For each plate to be labeled, aspirate the growth medium and wash twice with 3 ml PBS.
6. Add 2 ml equilibrated labeling medium/plate, and incubate for 30 min (*see* **Note 15**). During this time, prepare and sterilize a 2 mg/ml solution of either emetine (pulse-labeling) or anisomycin (chase-labeling).
7. Add 100 µl of the appropriate inhibitor of cytoplasmic translation (final concentration of 100 µg/ml) to each plate and incubate for 5 min.
8. Add 400 µCi of EasyTag labeling mixture to each plate (final concentration of 200 µCi/ml) and incubate for 60 min.
9. Remove labeling mixture from cells and dispose off it according to University guidelines for the handling of radioisotopes. For pulse-labeling, add 5 ml of equilibrated DMEM+10% FBS/plate and return to the incubator for 10 min. For chase-

labeling, wash cells once with either DMEM+10% FBS or with PBS, and chase in DMEM+10% FBS (5 ml/plate) for up to 17.5 h (*see* **Note 16**).

10. Wash cells three times with PBS (*see* **Note 17**).
11. Using the cell lifter, scrape cells in 0.7–0.8 ml ice-cold PBS and then use a pipette to transfer the entire volume to an Eppendorf tube. Repeat with an additional 0.7–0.8 ml ice-cold PBS to collect cells remaining on the plate, and transfer to the same Eppendorf tube (total volume of ~1.5 ml) (*see* **Note 18**).
12. Collect cells by centrifugation at 1,500 × g for 10 min at 4°C.
13. Aspirate PBS and resuspend the pellet in 200 µl ice-cold PBS. From this point onward, keep cells on ice until they are resuspended in gel loading buffer. Samples may now be stored at –80°C for later use or the procedure may be continued (*see* **Note 19**).

3.1.2. Sample Preparation

1. Use the Micro-BCA™ Protein Assay Kit to determine the protein concentration of each sample. Duplicates of 5 and 10 µl are used, and the protein concentration for each of the duplicates must be within 10–15% of each other, otherwise the measurement should be repeated.
2. For each sample, spin down the desired amount of protein (usually 50 µg) by centrifugation at >10,000 × g for 20 min at 4°C (*see* **Note 20**).
3. Resuspend the pellet in 10 µl of 2X gel loading buffer (room temperature) and 10 µl of double-distilled water.
4. Sonicate samples for 3–8 s at an output control of 60.
5. Spin samples at room temperature for 10–15 min at >10,000 × g or until the bubbles resulting from sonication have disappeared.

3.1.3. SDS-PAGE

1. These instructions assume the use of a PROTEAN II xi gel system from Bio-Rad Laboratories. Rinse the glass plates, spacers, combs, and casting stand gaskets several times with deionized, then with double-distilled water, followed by a final rinse in 70–95% ethanol. Air-dry.
2. Prepare 12 ml of a 15% gel mixture by combining 6 ml acrylamide/bisacrylamide solution, 3 ml of 4X separating buffer, 2.9 ml of double-distilled water, 60 µl of 10% APS, and 6 µl of TEMED. Prepare 12 ml of a 20% gel mixture by combining 8 ml acrylamide/bisacrylamide solution, 3 ml of 4X separating buffer, 0.9 ml of double-distilled water, 60 µl of 10% APS, and 6 µl of TEMED.

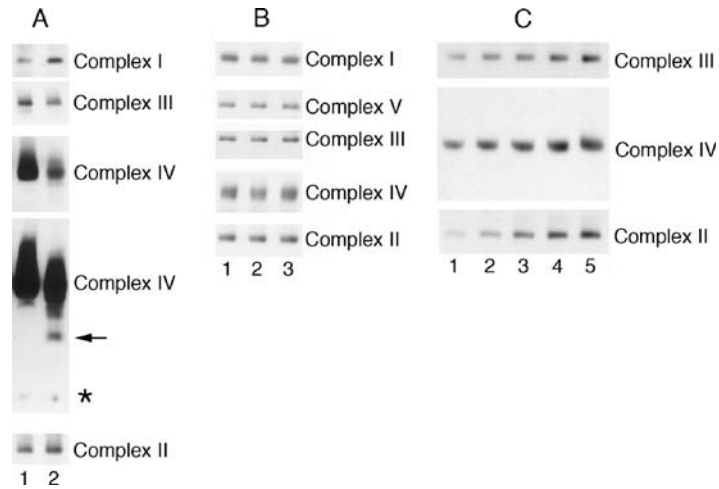

Fig. 10.2. *Representative BN-PAGE gels of samples prepared by differential digitonin permeabilization. Panel A*: Control human fibroblasts either without (1) or with (2) stable expression of a short-hairpin RNA that results in the knockdown of COX11, an assembly factor critical to Complex IV biogenesis. While the assembly of Complexes I, II, and III is unaffected, there is a significant accumulation of both monomeric COXIV (asterisk) and the S2 assembly intermediate (*arrow*). *Panel B*: The abundance of OXPHOS complexes in mouse spinal cord extracts (1–3). *Panel C*: The levels of Complexes II, III, and IV in HEK293 cells exposed to an increasing digitonin to protein ratio (1–5:0.2–1.6 mg digitonin:mg protein). In all panels, a total of 10 μg of protein was loaded per lane.

3. Using the WIZ Peristaltic Pump at its maximum flow rate, pour a 1.0-mm thick, 15–20% gradient gel by using the entire volume (24 ml) of the 15% and 20% gel solutions (*see* **Fig. 10.3**, for detailed instructions). Overlay the gradient gel with double-distilled water.
4. Once the gradient gel has polymerized, pour off the water overlay and dry the area above the gel with Whatman paper. Prepare the stacking gel by mixing 1.04 ml of acrylamide/bisacrylamide solution, 2.5 ml of 4X stacking buffer, 6.5 ml of double-distilled water, 50 μl of 10% APS, and 10 μl TEMED, and pour it on top of the separating gel until it begins to overflow. Insert the comb and allow the stacking gel to polymerize (*see* **Note 21**).
5. Once the stacking gel has set, remove the comb by pulling it straight up slowly and gently. Rinse the wells three times with double-distilled water.
6. Assemble the electrophoresis unit and add the running buffer to the inner and outer chambers of the unit. Load the whole 20 μl of each sample in an individual well (*see* **Note 22**). Reserve at least one well for the pre-stained molecular weight markers (15–20 μl/well).

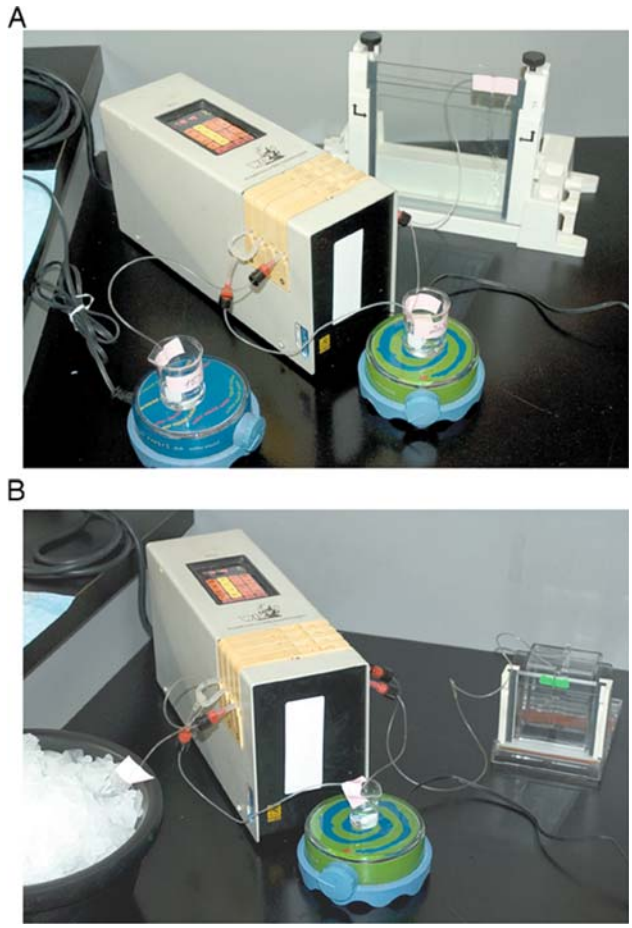

Fig. 10.3. *Organization, assembly, and casting of translation and BN-PAGE gels.* Clean and assemble the components required to cast either gel type (see relevant Methods sections for detailed instructions). Run double-distilled water through the WIZ Peristaltic Pump for roughly 5 min at a flow rate of 99 to ensure that the tubing is clean. Completely empty the tubing of all double-distilled water, and secure the relevant lines of tubing to either the beakers or the gel casting apparatus using tape, as shown in panels A (translation gel setup) and B (BN-PAGE gel setup). While the organization of the tubing does not differ between the two setups, note that the low percentage solution for the BN-PAGE gel is kept on ice and is not stirred while the gel is poured. Once the tubing is secured to both beakers, add the lowest percentage acrylamide solution to the left most beaker. Turn on the pump set to a flow rate of maximum for translation gels and 75 for BN-PAGE gels, and let the low percentage solution flow through the tubing until it almost reaches the beaker that will contain the highest percentage solution. Immediately add the high percentage solution to the right most beaker, and exhaust the entire volume of both solutions prior to stopping the pump. Overlay gently with water and allow at least 1 h for polymerization.

7. Complete the assembly of the electrophoresis unit and connect to a power supply. Run the gel at 10 mA for 16–16.5 h or until the lowermost (usually 11 kDa) molecular weight marker is at 1 cm from the bottom of the glass plates.

3.1.4. Generation and Analysis of the Data

1. At the end of the run, disconnect the electrophoresis unit from the power supply and disassemble it. Separate the glass plates sandwiching the gel by vigorously twisting one of the spacers. Remove and discard the stacking gel, and cut one corner of the separating gel to follow its orientation.
2. Rinse the gel by submerging it in a vessel containing double-distilled water, and transfer it by hand to a piece of thick filter paper cut to the dimensions of the gel. Cover the gel with Saran Wrap.
3. Dry gel under vacuum at 60°C for 1 h by using the SGD2000 Digital Slab Gel Dryer or equivalent.
4. Expose to a phosphorimager cassette for at least 3 days, then scan with the Storm 840 Gel and Blot Imaging System. Analyze the resultant image with the help of the ImageQuant TL Software. For characteristic patterns of mitochondrial translation analyzed by pulse-chase labeling and visualized by this method, *see* **Fig. 10.1**.

3.2. BN-PAGE

3.2.1. Cell Culture and Sample Preparation

1. Harvest a confluent 100-mm plate of cells by washing once in PBS, incubating in 4 ml of trypsin for 10 min, and neutralizing with an equal volume of medium (*see* **Note 23**).
2. Following neutralization, pellet the cells in a 15-ml Falcon tube, resuspend the pellet in 1 ml ice-cold PBS, transfer to an Eppendorf tube, and spin at 14,000 × g for 2 min at 4°C.
3. Resuspend the pellet in an arbitrary volume of ice-cold PBS and quantify the protein concentration using the Bradford assay.
4. Re-pellet the cells and resuspend in ice-cold PBS to a final concentration of 5 mg/ml for human fibroblasts (*see* **Note 24**).
5. Add an equal volume of digitonin at 4 mg/ml, mix the tube twice by inversion, and incubate on ice for 10 min.
6. Dilute with ice-cold PBS to a final volume of 1.5 ml, and spin for 10 min at 10,000 × g at 4°C.
7. Remove supernatant without disturbing the pellet, and wash it gently with 1 ml ice-cold PBS to completely remove residual digitonin.
8. Add BN sample buffer at half the volume that was initially required to resuspend the cell pellet at 5 mg/ml, and add lauryl maltoside at 1/10th of the BN buffer volume (*see* **Note 25**).

9. Resuspend pellet carefully by pipetting up and down 10–20 times, taking care not to foam the detergent (*see* **Note 26**).
10. Following a 20 min extraction on ice, spin at 20,000 × g for 20 min at 4°C.
11. Carefully remove the supernatant, transfer to a new Eppendorf tube, and quantify the protein concentration using the Bradford assay (*see* **Note 27**).
12. Add a volume of SBG that corresponds to half the volume of lauryl maltoside used in Step 8, and store at –20°C (*see* **Note 28**).

3.2.2. BN-PAGE

1. Prepare the glass plates, spacers, combs, and casting stand gaskets for either the Bio-Rad Mini-Protean II or Mini-Protean 3 gel system as described in **Section 3.1.3.**
2. Prepare 10 ml of a 6% gel mixture in a 15 ml-Falcon tube by combining 3.3 ml of 3X gel buffer, 1.2 ml of AB mix, and 5.44 ml of double-distilled water. Prepare the same volume of a 15% gel mixture in another 15-ml Falcon tube by combining 3.3 ml of 3X gel buffer, 3.0 ml of AB mix, 1.68 ml of double-distilled water, and 2 ml of 87% glycerol. Chill both solutions on ice for at least half an hour.
3. Add 60 µl of 10% APS and 4 µl of TEMED to the 6% gel mixture, and 10 µl of 10% APS and 2 µl of TEMED to the 15% gel mixture. Mix both solutions by inversion several times, and place on ice.
4. Set the WIZ Peristaltic Pump to a flow rate of 75, and pour a 1.0-mm thick, 6–15% gradient gel using 2.8 and 2.3 ml of the 6 and 15% gel stock solutions, respectively (*see* **Fig. 10.3**, for detailed instructions). Gently overlay the gradient gel with double-distilled water by gravity flow from 1-ml syringes with 24 gauge needles, and allow 1 h for polymerization (*see* **Note 29**).
5. While the gradient gel is polymerizing, prepare and chill 5 ml of stacking gel solution by combining 1.64 ml of 3X gel buffer, 0.4 ml of AB mix, and 2.87 ml of double-distilled water in a 15-ml Falcon tube.
6. Once the gradient gel has polymerized, pour off the overlay and use a Kimwipe to ensure complete removal of all residual water. Add 60 µl of 10% APS and 6 µl of TEMED to the stacking gel solution, mix by inversion several times, and pour it on top of the separating gel until it begins to overflow. Insert a 15-well comb.
7. Upon polymerization of the stacking gel, gently remove the comb by slowly pulling it straight up. Rinse the wells three times with colorless cathode buffer.

8. Add blue cathode buffer to the wells, and assemble the portion of the electrophoresis unit that will form the cathode (i.e., inner) chamber. Load wells with equal amounts of protein, while reserving at least one well for the molecular weight markers (*see* **Note 30**).
9. Insert the cathode chamber into the gel tank, and fill it with blue cathode buffer. Completely fill the gel tank (i.e., outer chamber) with anode buffer (*see* **Note 31**), fully assemble the electrophoresis unit, and connect to a power supply. Run the gel for 30–45 min at 35 V. Increase to 75 V for another half an hour and to 90–100 V for the remainder of the run.
10. Once the dye front is one third of the way through the gradient gel, stop the run and replace the blue cathode buffer with colorless cathode buffer (*see* **Note 32**). Continue electrophoresis until the dye front reaches the bottom of the gradient gel.

3.2.3. Western Blotting and Immunodetection

1. While the gel is running, cut six pieces of Whatman paper and nitrocellulose membrane to the exact dimensions of the gel(s) to be transferred.
2. Equilibrate the membrane in transfer buffer shortly before the end of the run. At the end of the run, disconnect the electrophoresis unit from the power supply and disassemble it. Separate the glass plates sandwiching the gel by twisting one of the spacers. Remove and discard the stacking gel, and cut the bottom right-hand corner of the gradient gel to mark its orientation.
3. Leaving the gel on the glass plate to which it has adhered, fully immerse it in a vessel containing transfer buffer, and gently rock it back and forth until it physically separates from the plate.
4. Prepare the apparatus for transfer by wetting a piece of Whatman paper and placing it on the cathode plate of the transfer apparatus. Carefully remove all bubbles by rolling a borosilicate tube over the Whatman paper. Dab away excess transfer buffer with Kimwipes as required (*see* **Note 33**).
5. Repeat Step 4 with two more pieces of Whatman paper and finally with the nitrocellulose membrane.
6. Handling the gel by its bottom end, carefully place it on the membrane. Remove any bubbles between it and the membrane by very gently rolling the borosilicate tube over the gel as many times as necessary.
7. Repeat Step 4 three more times.

8. Wet the anode plate with double-distilled water, fully assemble the apparatus, and transfer for 1 h at a constant milliamperage of 0.8 mA/cm^2 of nitrocellulose membrane.
9. At the end of the transfer, disassemble the apparatus, discard the gel(s), and place the nitrocellulose membrane in a vessel containing an ample volume of TBST.
10. Shake the membrane for 5 min, replace TBST with blocking solution, and continue shaking at room temperature for at least 1 h.
11. Replace the blocking solution with that containing the primary antibody solution and rock overnight at 4°C (*see* **Note 34**).
12. The following day, remove the primary antibody and wash the membrane six times for 5 min per wash with shaking at room temperature using ample volumes of TBST.
13. Incubate the membrane for 1 h in the secondary antibody solution, and repeat washes as outlined in Step 12.
14. Combine both ECL reagents with double-distilled water such that they each represent 1/20th of the final volume (10–20 ml total). Remove TBST from the vessel as completely as possible and replace with the ECL solution. Rock for 1 min. Using a pair of forceps, dab the membrane against the side of the vessel to remove excess ECL solution, and place it in a transparent acetate leaflet. Remove all bubbles with the help of a borosilicate tube.
15. Once in the darkroom, take multiple exposures of the membrane and develop the film.
16. Return the membrane to a vessel containing TBST. If stripping is not required, repeat Steps 10–15 with a different primary antibody. Should stripping be necessary, incubate the membrane in stripping buffer for 30 min while gently rocking in a water bath set at 50°C. Promptly remove stripping solution and wash the membrane extensively with TBST (4X 15 min) while shaking at room temperature. Repeat Steps 10–15 (*see* **Note 35**).

4. Notes

1. Certain formulations of DMEM without methionine and cysteine contain sodium pyruvate, while others do not. Check before adding!

2. Pure (^{35}S) methionine results in the strongest signal and the best signal/noise ratio. However, EasyTag mixture of (^{35}S) methionine and cysteine is considerably less expensive and gives a comparable result (approximately 75% of the signal intensity compared with pure (^{35}S) methionine).
3. Add warm medium to the CAP powder and incubate in a water bath at 37°C with occasional vortexing to help dissolve the powder.
4. Commercially available sources of digitonin contain impurities that affect its solubility in aqueous solutions. Full dissolution of digitonin in PBS requires boiling for 5 min. The solution is then cooled on ice and is stable for approx. 4 h. It should not be reused for preparation of samples on subsequent days.
5. The addition of protease inhibitors is not required when preparing native extracts from cultured cells.
6. Lauryl maltoside requires minimal heating or vortexing to go into solution. While it is stable indefinitely at 4°C, its efficacy declines with time. As a result, it should be made fresh the day that samples are prepared to ensure the most consistent results.
7. The use of SBG from other commercial sources results in a considerable increase in the non-specific background during Western blotting and should therefore be avoided.
8. In the event that the AB mix precipitates during storage, heat at 42°C and vortex periodically until it goes back into solution.
9. Cathode and anode buffers can be stored indefinitely at room temperature once they have been prepared.
10. High molecular weight markers are resuspended in 100 µl BN sample buffer, followed by the addition of 10 µl of 5% SBG. Loading 5–10 µl per lane allows for visualization of all five markers by Coomassie staining; however, only a subset of these are visible by Ponceau staining if they are transferred onto a nitrocellulose membrane.
11. We have found that this unbuffered form of TBS helps with consistent immunodetection of OXPHOS complexes. This may be attributable to the exposure by high pH of epitopes that are otherwise either partially or fully masked in this native gel system.
12. The stripping solution is supplemented with β-mercaptoethanol immediately prior to its use.
13. Starting with less than a 75% confluent plate might result in insufficient protein for SDS-PAGE analysis. However, cells must also be less than 100% confluent, as they should still be able to divide during radiolabeling.

14. Label a maximum of six plates at a time, otherwise it will be difficult to respect the required timing for several steps of the procedure.
15. Throughout the labeling procedure it is important that individual plates be placed directly on the shelf of the incubator, rather than stacked on top of each other. This ensures that all the plates have an equal opportunity to equilibrate in terms of temperature and CO_2 concentration.
16. The purpose of the short chase of 10 min in pulse-labeling is to allow the ribosomes to finish translating any radiolabeled proteins; otherwise, any "hot" protein shorter than the full-length species will run at a different size. While the short chases can be universally done in DMEM+10%FBS, longer chases should be done in cell-specific medium (e.g., chase myoblasts in myoblast-specific medium).
17. Be gentle when washing cells loosely attached to the plate, such as large myotubes or certain transformed cell lines.
18. Alternatively, in the case of myotubes, an enriched population of fused cells can be obtained by selective trypsinization: trypsinize cells for about 2 min or until fused cells start lifting (unfused myoblasts will take at least 5 min to trypsinize). Dilute trypsin by adding 5 ml PBS to the plate and transfer trypsinized cells to a 15-ml Falcon tube. Rinse plate with another 5 ml PBS and add to the same 15-ml tube. Collect cells by centrifugation at 1,500 × g for 5 min. Aspirate PBS, then resuspend pellet in ~1.5 ml cold PBS and transfer to an Eppendorf tube.
19. Remember to dispose appropriately of all materials that come in contact with the radioisotope: pipettes, cell plates, Eppendorf tubes, pipette tips, cell lifters, etc.
20. Especially when labeling a transformed cell line for the first time, run 25 and 50 μg of total cellular protein to verify the linearity of the resulting signal; 50 μg of total cellular protein should be within the linear range for most un-transformed cells.
21. This gel system allows the use of 15- and 20-well combs. While the 20-well comb has the obvious advantage of a higher number of samples per run, the 15-well comb will result in better definition of the bands and a higher resolution between lanes, both of which improve the quantification of the signal.
22. To save time, prepare the running buffer while the separating gel polymerizes. Likewise, start preparing the samples after pouring the stacking gel.
23. One confluent 100-mm plate of human fibroblasts or myoblasts will yield sufficient material to prepare and analyze a sample at least twice, assuming that 10–20 μg of total protein are loaded per lane.

24. If a different cell type or the same cell type from another species is being prepared for BN-PAGE, it is important to ensure that the digitonin to protein ratio that is used achieves maximal enrichment for OXPHOS complexes without promoting either their dissociation or degradation. In our experience, a digitonin to protein ratio of 0.8 and 1.2 is appropriate for generating enriched mitoplasts from human fibroblasts and myoblasts, respectively.
25. In our experience, roughly half of the total cellular protein is depleted upon treatment of human fibroblasts (and myoblasts) with digitonin. The purpose of reducing the volume of BN buffer used in the solubilization of the enriched mitoplast pellet reflects a desire to maintain a protein concentration of roughly 2–4 mg/ml at this stage of the isolation. If a different digitonin to protein ratio is used in the preparation of enriched mitoplasts from other cell lines, the volume of BN buffer to be used should also be adjusted accordingly.
26. It is impossible to generate a homogeneous solution when resuspending the pellet. Once the solution has been repeatedly pipetted up and down as described in Step 9, small, tight fragments of "insoluble" material should be visible. Do not use more vigorous means of solubilizing the pellet (e.g., homogenizing) since this may promote the dissociation of OXPHOS complexes.
27. The final protein concentration should be between 1 and 3 mg/ml. Lower protein concentrations may result in the dissociation of OXPHOS complexes, while higher protein concentrations may lead to their anomalous or inconsistent migration due to partial release from either the inner mitochondrial membrane or higher order complexes (i.e., supercomplexes).
28. Freezing at –20°C only preserves the integrity of OXPHOS complexes for 1–2 months. For long-term storage, samples may be kept indefinitely at –80°C.
29. The volumes specified for pouring the gradient and stacking gels are sufficient to cast up to three gels. These may be scaled either up or down based on individual needs; however, no more than four gradient gels should be prepared from the same solutions as there is an increased risk of polymerization within the pump tubing.
30. Loading 10–20 μg of total protein per lane will permit for the immunodetection of all five OXPHOS complexes. Samples are loaded prior to filling the cathode chamber with blue cathode buffer due to the extreme difficulty one otherwise has visualizing the wells.

31. It is very difficult to generate a perfectly sealed cathode chamber. Completely filling the gel tank with anode buffer therefore serves to minimize the potential leaking of cathode buffer into the anode chamber.
32. The cathode buffer is changed from blue to colorless at this stage of electrophoresis to minimize the amount of Coomassie that remains in the gel at the end of the run. This will not affect the electrophoretic mobility of the proteins and greatly enhances the signal to noise ratio upon Western blotting.
33. The presence of excess transfer buffer will affect the quality of protein transfer to the membrane. Transferring more than two gels at a time using this particular system will also adversely affect the quality of transfer.
34. The monoclonal antisera raised against structural subunits of OXPHOS can be repeatedly frozen and thawed, thus extending their lifespan, if their stock solution is diluted with TBST supplemented with BSA as opposed to milk. The only exception is anti-ATPase α, which, even in BSA, retains its immunoreactivity for a maximum of 2–3 freeze/thaw cycles.
35. Stripping is only necessary if the complexes to be detected are of similar sizes (e.g., Complexes III and V), and repeated stripping should be avoided since it will result in an unwanted loss of protein from the membrane. Sequential blotting for Complexes IV, V, and II, followed by stripping of the membrane and subsequent blotting for Complexes I then III is therefore preferable.

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