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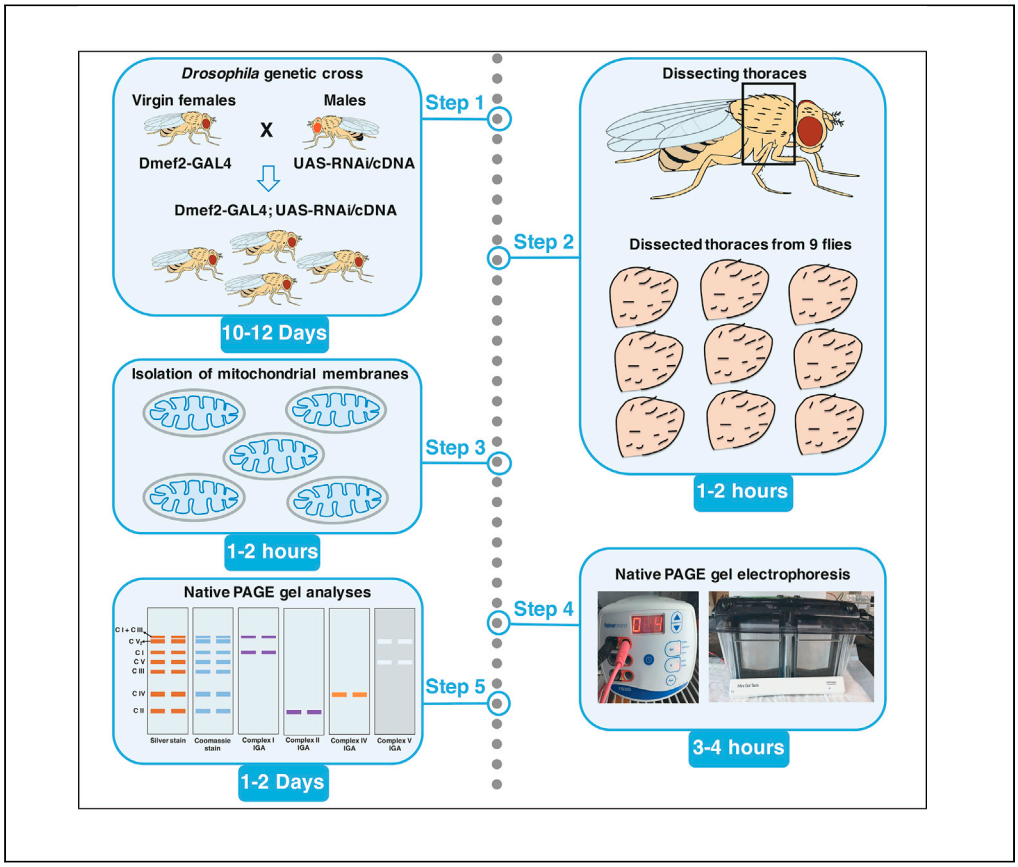
Analyzing the integrity of oxidative phosphorylation complexes in *Drosophila* flight muscles



Drosophila flight muscles are highly enriched with mitochondria and have emerged as a powerful genetic system for studying how oxidative phosphorylation (OXPHOS) complexes are assembled. Here, we describe a series of protocols for analyzing the integrity of OXPHOS complexes in *Drosophila* via blue native polyacrylamide gel electrophoresis (BN PAGE). We have also included protocols for the additional steps that are typically performed after OXPHOS complexes are separated by BN PAGE, such as Coomassie staining, silver staining, and in-gel OXPHOS activities.

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Highlights

Blue native polyacrylamide gel electrophoresis (BN PAGE) separates OXPHOS complexes

OXPHOS assembly in *Drosophila* flight muscles can be assessed with BN PAGE

OXPHOS activities can be assessed by in-gel activity assays

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Analyzing the integrity of oxidative phosphorylation complexes in *Drosophila* flight muscles

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SUMMARY

***Drosophila* flight muscles are highly enriched with mitochondria and have emerged as a powerful genetic system for studying how oxidative phosphorylation (OXPHOS) complexes are assembled. Here, we describe a series of protocols for analyzing the integrity of OXPHOS complexes in *Drosophila* via blue native polyacrylamide gel electrophoresis (BN PAGE). We have also included protocols for the additional steps that are typically performed after OXPHOS complexes are separated by BN PAGE, such as Coomassie staining, silver staining, and in-gel OXPHOS activities.**

For complete details on the use and execution of this protocol, please refer to Murari et al. (2020).

BEFORE YOU BEGIN

The flight muscles in *Drosophila* have frequently been exploited to study mitochondrial dynamics and how various mitochondrial proteins influence overall organismal physiology and aging (Deng et al., 2008; Garcia et al., 2017; Owusu-Ansah et al., 2013; Rana et al., 2017; Rera et al., 2011; Ulgherait et al., 2014). Here, we describe a series of approaches for studying the assembly of OXPHOS complexes in *Drosophila* flight muscles. As *Drosophila* thoraces are enriched with mitochondria, we use mitochondria from 10 fly thoraces to check the integrity of OXPHOS complexes by BN-PAGE, followed by silver staining and Coomassie staining. For in-gel activity assays of OXPHOS complexes, we use mitochondria isolated from 20 fly thoraces. While we focus on the flight muscles, the techniques we describe can be adapted for use in other tissues, such as the brain, gut, and larval muscles. This protocol assumes the reader has a working knowledge of *Drosophila* genetics and the OXPHOS system.

Fly husbandry

Timing: Approximately 2 weeks

1. Collect virgins of the appropriate genotype.
2. Keep the virgins in a fly incubator at 25°C for 3–5 days prior to setting up the crosses.

⚠ CRITICAL: Before setting up the crosses, check that the female flies to be used are actually virgins, by ensuring that no larvae are present in the vials housing the female flies.

3. Set up each cross with approximately 9 virgins (3–5 days old) and 3 adult male flies of the appropriate genotype in a fly food vial. For best results, use male flies that are 2–3 days old.

4. Set up the crosses at 25°C in a Forma environmental chamber or any suitable fly incubator with a 12 h day/12 h night cycle.
5. Let the flies lay eggs for at least 48 h at 25°C.
6. Transfer the flies into fresh fly food and return both vials (both the new and previous vial that was used to start the cross) to the 25°C incubator.
7. Repeat steps 5 and 6 to obtain 3 sets of crosses.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Sucrose	Fisher Scientific	S5-500
Magnesium Chloride ($MgCl_2$)	Fisher Scientific	M33-500
Tris Base	Fisher Scientific	BP152-25
Bis-Tris	Fisher Scientific	BP301-100
Tricine	Fisher Scientific	BP315-100
Sodium Phosphate Monobasic Anhydrous (NaH_2PO_4)	Fisher Scientific	S397-500
Sodium Phosphate Dibasic Anhydrous (Na_2HPO_4)	Fisher Scientific	S375-500
Hydrochloric Acid (HCl)	Fisher Scientific	SA55
Methanol	Fisher Scientific	A412-4
Glacial Acetic Acid	Fisher Scientific	A38-500
Digitonin (−20°C)	Sigma-Aldrich	D141-500MG
G-250 Sample Additive (Blue Dye) (4°C)	Invitrogen	BN2004
Sample Buffer (4°C)	Invitrogen	BN2003
20× Cathode Buffer Additive	Invitrogen	BN2002
Halt Protease Inhibitor (4°C)	Thermo Scientific	PI78430
Bovine Serum Albumin (4°C)	Fisher Scientific	BP9703100
Nitrotetrazolium Blue Chloride (NBT) (4°C)	Sigma-Aldrich	N6876-500MG
Nicotinamide Adenine Dinucleotide Hydrate (NADH) (−20°C)	Sigma-Aldrich	N7004-1G
Adenosine Triphosphate (ATP) (4°C)	Acros Organics	AC102800100
Disodium Succinate	Sigma-Aldrich	W327700
Phenazine Methosulfate (−20°C)	Sigma-Aldrich	P9625-1G
Diaminobenzidine (DAB) (4°C)	Sigma-Aldrich	D5637-5G
Cytochrome C (−20°C)	Sigma-Aldrich	C2506-100MG
Glycine	Fisher Scientific	G46-500
Magnesium Sulfate Anhydrous ($MgSO_4$)	Fisher Scientific	M65-500
Lead Nitrate [$Pb(NO_3)_2$]	Sigma-Aldrich	228621-100G
Bradford 1× Dye Reagent	Bio-Rad Laboratories	5000205
Experimental models: Organisms/strains		
D. melanogaster strain expressing GAL4 in muscles under the control of the Mef2 promoter. yw[1118]; Dmef2-Gal4	(Ranganayakulu et al., 1996)	Dmef2-gal4
D. melanogaster strain expressing temperature-sensitive GAL80 under the control of the alphaTub84B promoter. w [*]; P{w{+mC] =tubPGAL80[ts]}10; TM2/TM6, Tb[1]	Bloomington Drosophila Stock Center	BDSC 7108; Flybase: FBti0027799
D. melanogaster strain expressing a transgenic RNAi construct to dErv1 (Alr). y^1 sc* v^1 sev^{21}; P {TRiP.HMC03697}attP40	Bloomington Drosophila Stock Center	BDSC 65088; Flybase: FBst0065088
D. melanogaster strain expressing a transgenic RNAi construct to AIF. w^{1118}; P{GD822}v2544	Vienna Drosophila Resource Center	VDRC v2544; Flybase: FBst0455887
D. melanogaster strain, wild type used as a control. w^{1118}	Bloomington Drosophila Stock Center	BDSC 3605; Flybase: FBst0003605
Critical commercial assays		
Colloidal Blue Staining Kit	Invitrogen	LC6025
SilverXpress Silver Staining Kit	Invitrogen	LC6100

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Light Contrast Microscope	Olympus	SZ61
Forceps	Fine Science Tools	11251-10
Power Supply	Fisher Scientific	FB300
Bullet Blender	Next Advance	BBX24B
Zirconium Oxide Beads	Next Advance	ZROB05
NativePAGE 3%–12% Bis-Tris Gel	Invitrogen	BN1003BOX
Forma Environmental Chamber	Fisher Scientific	13-067-066
ChemiDoc Imaging System	Bio-Rad Laboratories	17001401
Mini Gel Tank	Invitrogen	A25977
LaserJet 200 color MFP	HP	CF145A#BGJ

MATERIALS AND EQUIPMENT

Preparing mitochondria isolation medium

Mitochondria isolation medium (MIM)

Reagent	Final concentration	Amount
1 M Sucrose	250 mM	250 mL
0.1 M $MgCl_2$	0.15 mM	1.5 mL
1 M Tris-HCl	10 mM	10 mL
ddH_2O		738.5 mL
Total		1000 mL

Aliquot in 50 mL portions in a falcon tube. Store up to 1 year @ −80°C.

Preparing 20× BN PAGE running buffer

Composition of the 20× BN PAGE running buffer

Reagent	Final concentration	Amount
Bis-Tris	1 M	209.2 g
Tricine	1 M	179.2 g
ddH_2O		Adjust volume to 1000 mL
Total		1000 mL

Store up to 6 months @ room temperature (25°C).

Preparing 1× BN PAGE anode buffer

Composition of the 1× BN PAGE anode buffer

Reagent	Final concentration	Amount
20× Running Buffer	50 mM	50 mL
ddH_2O		950 mL
Total		1000 mL

Store up to 6 months @ 4°C.

Preparing 1× BN PAGE cathode buffer

Composition of the 1× BN PAGE cathode buffer

Reagent	Final concentration	Amount
20× Running Buffer	50 mM	50 mL
20× Cathode Buffer Additive	0.1×	5 mL
ddH_2O		945 mL
Total		1000 mL

Store up to 6 months @ 4°C.

Preparing BN PAGE fixative solution

Composition of the BN PAGE fixative solution

Reagent	Final concentration	Amount
Methanol	40%	40 mL
Glacial Acetic Acid	10%	10 mL
ddH_2O		50 mL
Total		100 mL

Store up to 6 months @ room temperature (25°C).

Preparing BN PAGE staining solution

Composition of the BN PAGE staining solution

Reagent	Final concentration	Amount
Methanol	20%	20 mL
Stainer A (from Colloidal Blue Staining Kit)	20%	20 mL
Stainer B (from Colloidal Blue Staining Kit)	5%	5 mL
ddH_2O		55 mL
Total		100 mL

Store up to 1 month @ room temperature (25°C).

Preparing BN PAGE destaining solution

Composition of the BN PAGE destaining solution

Reagent	Final concentration	Amount
Glacial Acetic Acid	8%	80 mL
ddH_2O		920 mL
Total		1000 mL

Store up to 6 months @ room temperature (25°C).

Preparing 1% digitonin buffer

Composition of the 1% digitonin buffer (D)

Reagent	Final concentration	Amount
4× Sample Buffer	1×	62.5 µL
5% Digitonin	1%	50 µL
100× Protease Inhibitor	1×	2.5 µL
Ultrapure Water		135 µL
Total		250 µL

Storage conditions: Make just before you begin, and keep on ice.

Preparing G-250 blue dye

Composition of the blue dye (B)

Reagent	Final concentration	Amount
4× Sample Buffer	2.67×	100 μL
5% G-250 Sample Additive	0.42%	12.5 μL
Ultrapure Water		37.5 μL
Total		150 μL

Storage conditions: Make just before you begin, and keep on ice.

Preparing sensitizer solution

Composition of the sensitizer solution

Reagent	Final concentration	Amount
Sensitizer (from SilverXpress Silver Staining Kit)	Unknown	5 mL
Methanol	47.6%	100 mL
Ultrapure Water		105 mL
Total		210 mL

Storage conditions: Make just before you begin, and keep @ room temperature (25°C).

Preparing silver staining solution

Composition of the silver staining solution

Reagent	Final concentration	Amount
Stainer A (from SilverXpress Silver Staining Kit)	Unknown	5 mL
Stainer B (from SilverXpress Silver Staining Kit)	Unknown	5 mL
Ultrapure Water		90 mL
Total		100 mL

Storage conditions: Make just before you begin, and keep @ room temperature (25°C).

Preparing developer solution

Composition of the developer solution

Reagent	Final concentration	Amount
Developer (from SilverXpress Silver Staining Kit)	Unknown	5 mL
Ultrapure Water		95 mL
Total		100 mL

Storage conditions: Make just before you begin, and keep @ room temperature (25°C).

Preparing 0.1 M sodium phosphate dibasic solution

Composition of the 0.1 M sodium phosphate dibasic solution

Reagent	Final concentration	Amount
Sodium Phosphate Dibasic	0.1 M	7.1 g
Ultrapure Water		Adust volume to 500 mL
Total		500 mL

Store up to 1 year @ room temperature (25°C).

Preparing 0.1 M sodium phosphate monobasic solution

Composition of the 0.1 M sodium phosphate monobasic solution

Reagent	Final concentration	Amount
Sodium Phosphate Monobasic	0.1 M	6.0 g
Ultrapure Water		Adust volume to 500 mL
Total		500 mL

Store up to 1 year @ room temperature (25°C).

Preparing 0.1 M sodium phosphate buffer pH 7.2

Composition of the 0.1 M sodium phosphate buffer pH 7.2

Reagent	Final concentration	Amount
0.1 M Sodium Phosphate Dibasic Solution	0.1 M	100 mL
0.1 M Sodium Phosphate Monobasic Solution	0.1 M	Adjust pH to 7.2 by adding drops of the 0.1 M sodium phosphate monobasic solution to 100 mL of the 0.1 M sodium phosphate dibasic solution

Store up to 1 year @ room temperature (25°C).

Preparing complex I in-gel activity solution

Composition of the complex I in-gel activity solution

Reagent	Final concentration	Amount
10 mg/mL Nitrotetrazolium Blue	2.5 mg/mL	1.25 mL
40 mM NADH	0.136 mM	0.017 mL
10 mM Tris-HCl, pH 7.4	5 mM	2.5 mL
Ultrapure Water		1.233 mL
Total		5 mL

Storage conditions: Make just before you begin, and keep on ice.

Preparing complex II in-gel activity solution

Composition of the complex II in-gel activity solution

Reagent	Final concentration	Amount
1 M Sodium Succinate	20 mM	0.1 mL
20 mM Phenazine Methosulfate	0.2 mM	0.05 mL
10 mg/mL Nitrotetrazolium Blue	2.5 mg/mL	1.25 mL
10 mM Tris-HCl, pH 7.4	5 mM	2.5 mL
Ultrapure Water		1.1 mL
Total		5 mL

Storage conditions: Make just before you begin, and keep on ice.

Preparing complex IV in-gel activity solution

Composition of the complex IV in-gel activity solution

Reagent	Final concentration	Amount
1% Diaminobenzidine	0.05%	0.25 mL
0.83 mM Cytochrome C	0.05 mM	0.3 mL
100 mM Sodium Phosphate, pH 7.2	50 mM	2.5 mL
Ultrapure Water		1.95 mL
Total		5 mL

Storage conditions: Make just before you begin, and keep on ice.

Preparing complex V in-gel activity solution		
Composition of the complex V in-gel activity solution		
Reagent	Final concentration	Amount
1 M Magnesium Sulfate	14 mM	0.07 mL
10% Lead Nitrate	0.2%	0.1 mL
100 mM Adenosine Triphosphate	8 mM	0.4 mL
35 mM Tris Base + 270 mM Glycine, pH 8.4		4.43 mL
Total		5 mL
Storage conditions: Make just before you begin, and keep on ice.		

STEP-BY-STEP METHOD DETAILS

Collection and aging of flies

Timing: Approximately 1–2 days (excluding the variable period during which the flies are aged)

1. Following eclosure of the flies (after approximately 10 days at 25°C), collect male and female offspring.
2. Maintain collected flies in vials at a density of less than 25 flies per vial, and age them at 25°C until they reach a suitable time point.
3. While aging the flies, transfer them into new food 2–3 times per week. Discard the old vials.

Dissection of *Drosophila* thoraces

Timing: Approximately 1 h

4. Once the flies reach the desired age, anesthetize the flies on a CO_2 pad, and dissect 10 fly thoraces under a microscope using suitable dissection forceps.

Note: Anesthetized flies are dissected by severing both head and abdomen from the thorax. Save the thorax. Flies do not need to be in a buffer during dissection, and wings or legs do not have to be removed.

5. Place the dissected fly thoraces in an Eppendorf tube (without any buffer). Store immediately at −80°C until you are ready to perform the experiment.

Pause point: The frozen thoraces can be stored for at least a year at −80°C.

Isolation of mitochondrial membranes from thoraces

Timing: Approximately 1 h

6. Thaw an aliquot of the MIM (see materials and equipment) by incubating it in a 37°C water bath until it is almost completely defrosted. Remove and keep on ice until it is completely thawed .

⚠ CRITICAL: Check the MIM periodically to ensure it does not fully defrost while in the 37°C water bath. When there is little ice left take it out and place it in the ice bucket. Vortex briefly to mix thoroughly.

7. Obtain dissected thoraces from −80°C and keep on ice to defrost (Figure 1A).
8. Place the sample buffer, protease inhibitor, and blue dye in the ice bucket.

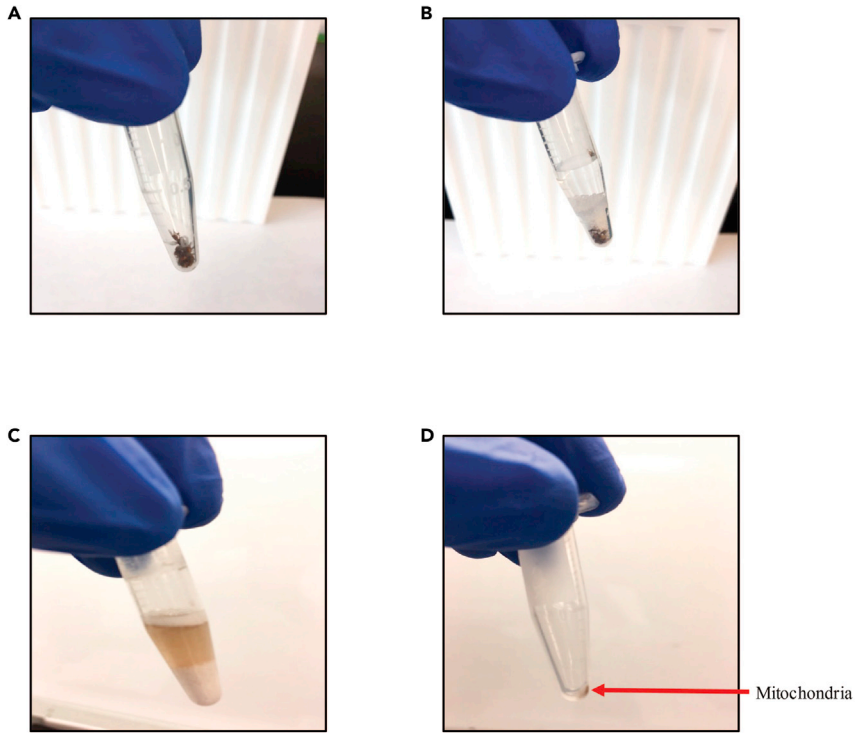

Figure 1. Mitochondria isolation from *Drosophila* flight muscles

Images showing fly thoraces before homogenization (A), fly thoraces with one scoop of beads, protease inhibitor and MIM (B), homogenized fly thoraces (C), and isolated mitochondrial pellet (D).

9. Add one scoop of Zirconium Oxide beads (equivalent to a bead volume of about 0.2 mL) and 0.5 mL of MIM containing 1× of Halt Protease inhibitors to each sample (Figure 1B).
10. Homogenize the samples in the bullet blender (BB) for 2 min, at a speed of 7 on the BB in the cold room (Figure 1C).

⚠ CRITICAL: Analyze the samples to see if chunks of the thorax remain in suspension. For optimal yield, the thorax must be homogenized to the point where the remains of the thorax are barely/slightly visible in the liquid.

11. Centrifuge the homogenized samples at 500 g (500 rcf) for 5 min at 4°C.
12. In the meantime, start defrosting the 5% digitonin suspension.
13. Following centrifugation for 5 min, transfer the supernatant into new Eppendorf tubes.
14. Centrifuge the supernatant again at 500 g for 5 min at 4°C.
15. Transfer the supernatant into new Eppendorf tubes and centrifuge at 5000 g for 5 min at 4°C.
16. Discard the supernatant and save the mitochondria-enriched pellet (Figure 1D).

BN PAGE gel tank preparation

Timing: Approximately 10–20 min

17. Prepare the gel tank as follows:
    a. Obtain gel plates (3%–12% gradient native gels) and the gel tank (Figure 2). Unwrap the plates, remove the comb and wash with Milli-Q water, and then with cathode buffer. Peel off the white strip and place it in the gel chamber and lock it.

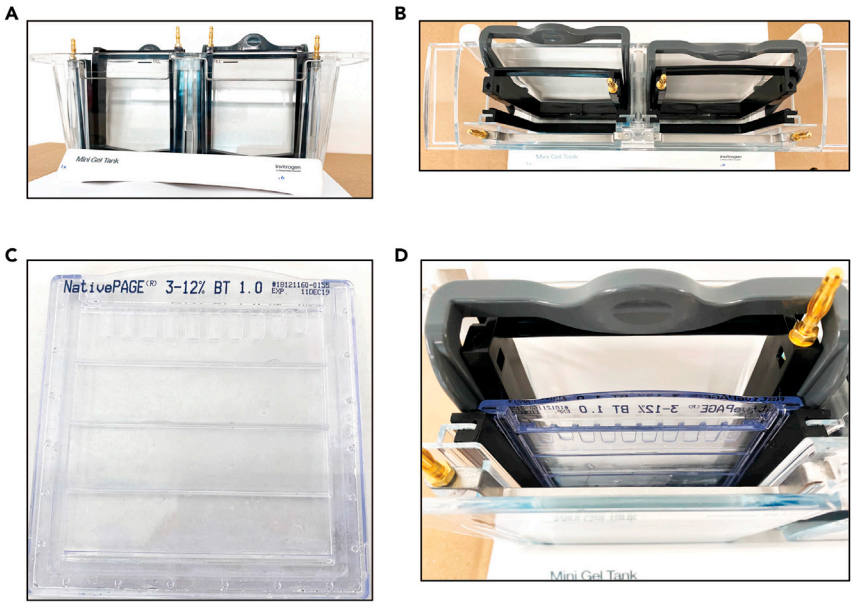

Figure 2. Setting up the gel tank for gel electrophoresis
Images showing gel tank prior to assembling the gel plate (side view) (A), gel tank prior to assembling the gel plate (top view) (B), 3%–12% gradient Native PAGE gel plate (C), one half of the gel tank after assembling the gel plate (D).

b. Add the anode buffer to the outer chamber, and cathode buffer to the inner chamber. Be sure to leave enough room for the wells to be visible. Using a 1000 μL pipette, add the cathode buffer to the wells.
c. After setting up the gel tank, place the gel tank in the cold room or any 4°C incubator.

Sample preparation for BN PAGE silver staining

Timing: Approximately 4 h

18. Solubilize the mitochondrial pellet (from step 16) by adding 25 μL of 1% digitonin buffer. Solubilize by pipetting up-and-down (see materials and equipment).
19. Incubate the solubilized mitochondrial pellet on ice for 15 min.
20. Centrifuge the samples at 20,000 g for 20 min at 4°C.
21. Save the supernatant, and transfer it into new Eppendorf tubes. Keep on ice.

Note: The supernatant will have the extracted OXPHOS complexes, while what remains of the membranes will be in the pellet.

22. Measure the protein concentration by Bradford method and normalize the samples to ascertain how much (volume) of each sample needs to be loaded in the gel for equal loading.
23. In a new Eppendorf tube, add 15 μL G-250 blue dye (see materials and equipment), 10 μL 1× sample buffer and 5 μL sample; mix gently.
24. Load the samples in wells based on the amounts given by the Bradford method calculations.
25. Run the gel using the FB300 Fisher Scientific power supply at 4°C.
26. For two gels, run at 300 V and 4 mA (keep milliamps constant) for approximately 3 h.

Silver staining of native gel

This method allows the OXPHOS complexes to be visualized. It is more sensitive than Coomassie staining.

Figure 3. Imaging of a silver-stained native gel by a Bio-Rad ChemiDoc Imaging system

Images of the Bio-Rad ChemiDoc instrument (A), program setting to capture a silver-stained native gel by the ChemiDoc Imager (B), and a silver-stained native gel captured by the ChemiDoc Imager (C).

Timing: Approximately 1–2 h

Note: All steps are at room temperature.

27. After gel electrophoresis is complete, remove the gel from the cassette, place in a container with the silver staining fixative solution (50% methanol and 10% acetic acid) and rotate on a shaker for 10 min.
28. Remove the fixative solution and add 100 mL of the sensitizer solution (105 mL of ultrapure water, 100 mL of methanol and 5 mL of Sensitizer) and keep on the shaker for 10 min.
29. Following sensitization, wash the gel twice with 200 mL of ultrapure water for 5 min each.
30. Then incubate the gel in the silver staining solution (90 mL of ultrapure water, 5 mL of Stainer A and 5 mL of Stainer B) for 15 min on the shaker.
31. Wash the gel twice with 200 mL of ultrapure water for 5 min each.
32. Develop the gel by adding the developer (95 mL of ultrapure water and 5 mL of Developer). Stop the reaction by adding the stopper solution once bands are visible.
33. After about 10 min, capture the image with the ChemiDoc Imaging System (Figures 3A–3C and 4A).

Sample preparation for BN PAGE Coomassie staining

Timing: Approximately 4 h

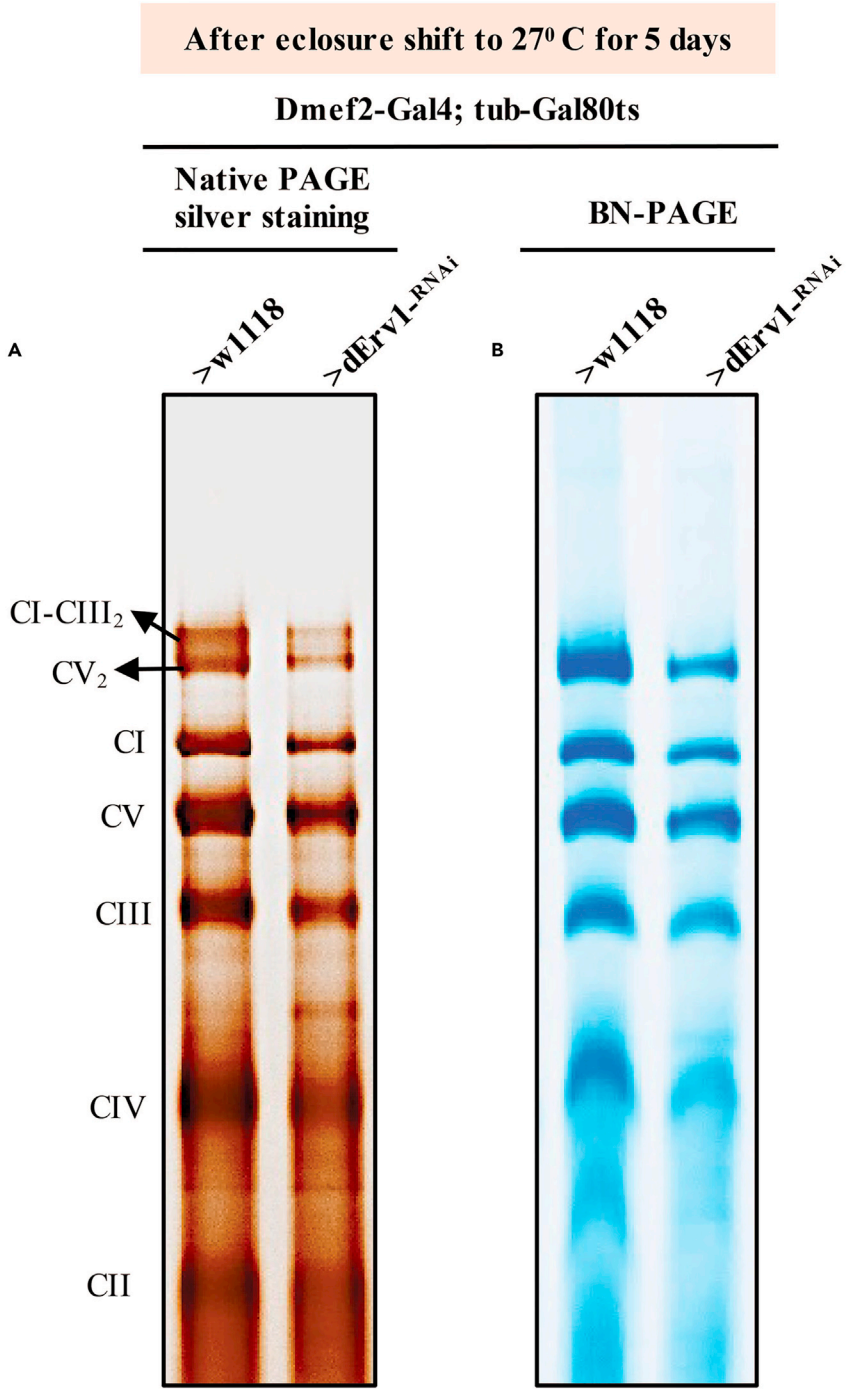

Figure 4. Analysis of OXPHOS complexes integrity by BN PAGE and silver staining of native gels

BN PAGE followed by silver staining of native gels containing OXPHOS complexes isolated from mitochondria from flight muscles of flies with the genotype indicated (A). BN PAGE and Coomassie staining analysis of native gels containing OXPHOS complexes isolated from mitochondria from flight muscles of flies with the genotypes indicated (B).

34. In a new Eppendorf tube add 7.5 μL of the blue dye and 15 μL of sample and mix gently.
35. Load the samples in wells based on the amounts given by the Bradford method calculations.
36. Run the gel using the FB300 Fisher Scientific power supply at 4°C. For four gels, run at 300 V and 8 mA (keep milliamps constant) for approximately 3 h.

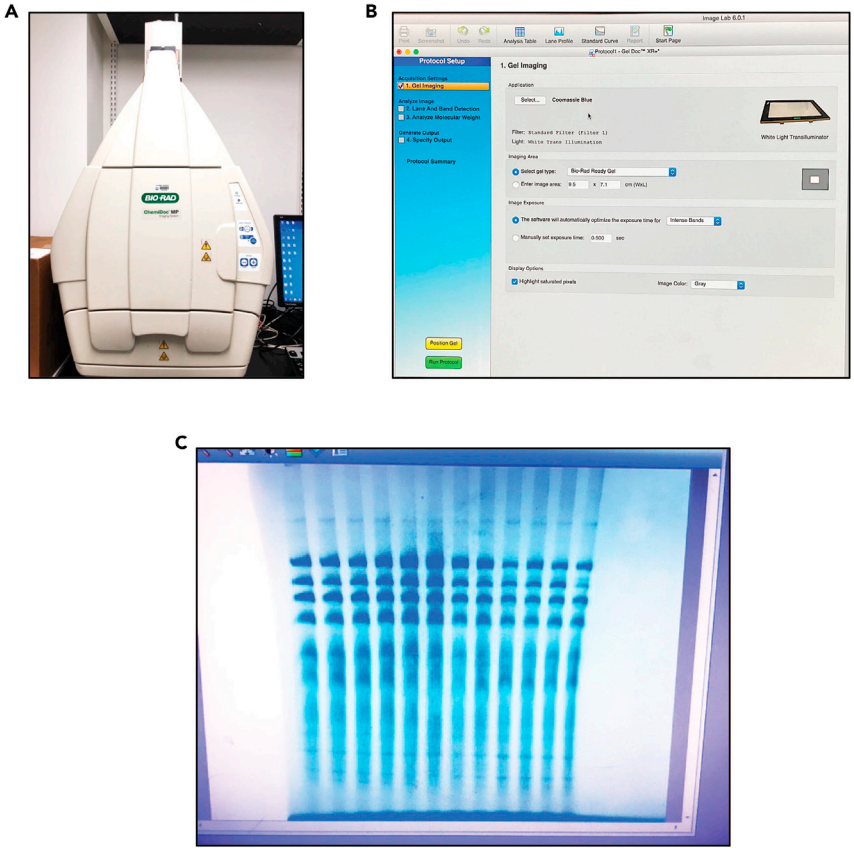

Figure 5. Imaging of a Coomassie-stained native gel by a Bio-Rad ChemiDoc Imaging System

Images of the Bio-Rad ChemiDoc instrument (A), program setting to capture the Coomassie-stained native gel by the ChemiDoc Imager (B), and a Coomassie-stained native gel captured by the ChemiDoc Imager (C).

Coomassie staining of native gel

(see materials and equipment).

This method allows the OXPHOS complexes to be visualized. It is less sensitive than silver staining.

Timing: Approximately 16 h

Note: All steps are at room temperature.

37. Following gel electrophoresis, remove the gel from the cassette, place in a container with the BN PAGE fixative solution (see materials and equipment) and rotate on the shaker for 30 min.
38. Remove the fixative solution, add 100 mL of the BN PAGE staining solution (see materials and equipment) and rotate on the shaker overnight (approximately 16 h).
39. Remove the staining solution and de-stain the gel by adding 100 mL of the de-stainer (see materials and equipment).
40. After the bands are visible, capture the image with the ChemiDoc Imaging System (Figures 5A–5C and 4B).

Because Coomassie does not denature proteins it is suitable for characterizing enzyme activities. In-gel enzyme assays make it feasible to identify enzymes in gels based solely on enzymatic activity. Typically, after a gel is run it is soaked in a buffer containing substrates for the enzyme to be

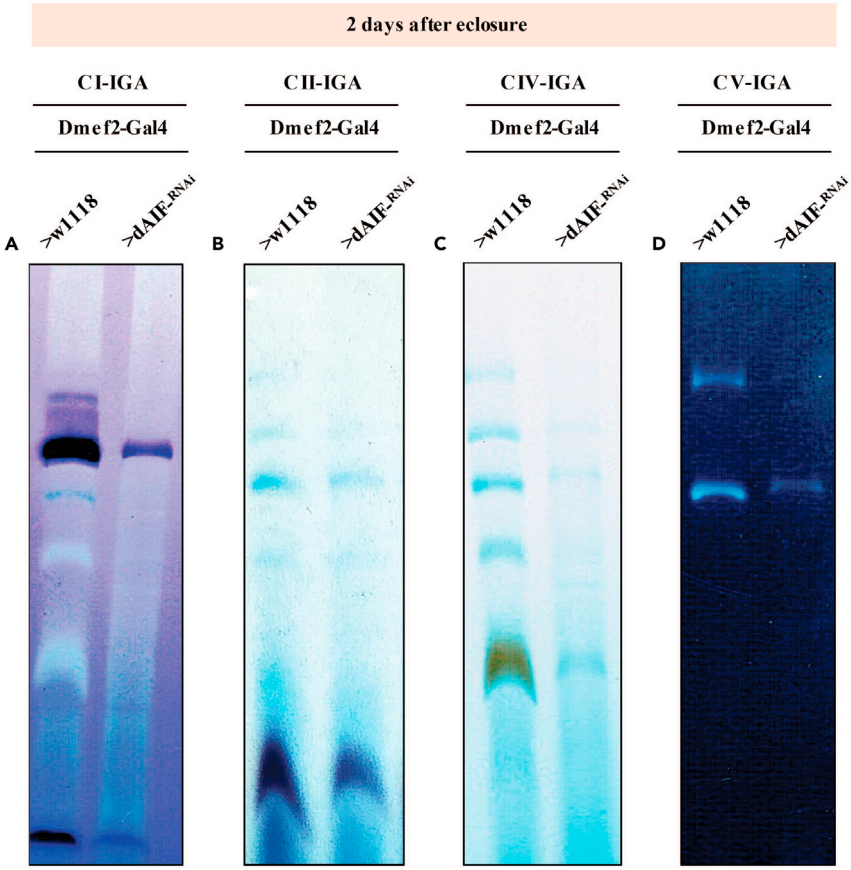

Figure 6. In-gel activity analyses of OXPHOS complexes in native gels

Complex I (A), Complex II (B), Complex IV (C), and Complex V (D) in-gel activity assays of OXPHOS complexes isolated from flight muscles with the genotypes indicated.

detected. Following incubation for a suitable period and a series of washes, the enzyme being assayed can be detected as a distinct colored band in the gel. Here, we describe protocols for visualizing CI, CII, CIV and CV activities of *Drosophila* OXPHOS complexes within blue native gels.

Sample preparation for in-gel activities of native gels (complex I, complex II, complex IV and complex V)

Timing: 2 days

Here, we describe the sample preparation and the specific steps for analyses of Complex I, II, IV, and V activities in native gels.

41. After steps 1–22, in a new Eppendorf tube add 20 μL of blue dye and 40 μL of sample and mix gently.
42. Load the samples in wells in four sets for CI, CII, CIV and CV in-gel activities based on the amounts given by the Bradford method calculations.
43. Run the gel using the FB300 Fisher Scientific power supply at 4°C. For two gels, run at 300 V and 4 mA (keep milliamps constant).
44. After the gel electrophoresis is complete, remove the gel from the cassette.

a. Complex I: Place the first gel in a container with Complex I in-gel activity solution (see materials and equipment), and place it on the shaker overnight (approximately 16 h) at 4°C. After the Complex I band is clearly visible, capture the image with a HP LaserJet scanner (Figure 6A).
b. Complex II: Place the second gel in a container with Complex II in-gel activity solution (see materials and equipment), and place it on the shaker overnight (approximately 16 h) at 4°C. After the Complex II band is clearly visible, capture the image with a HP LaserJet scanner (Figure 6B).
c. Complex IV: Place the third gel in a container with Complex IV in-gel activity solution (see materials and equipment), and place on the shaker overnight (approximately 16 h) at room temperature. After the Complex IV band is clearly visible, capture the image with a HP LaserJet scanner (Figure 6C).
d. Complex V: Place the fourth gel in a container with Complex V in-gel activity solution (see materials and equipment), and place on the shaker overnight (approximately 16 h) at room temperature. After the Complex V band is clearly visible, capture the image with a HP LaserJet scanner (Figure 6D).

EXPECTED OUTCOMES

This protocol provides the methodology for analyzing the integrity and activities of OXPHOS complexes in *Drosophila* tissues. Because BN PAGE preserves complexes in their native state it allows OXPHOS complexes and supercomplexes to be visualized in gels. As demonstrated here, BN PAGE can be followed by additional techniques such as Coomassie blue staining, silver staining and in-gel enzyme activity assays to expand its applications.

LIMITATIONS

The predominant factor that determines whether a BN PAGE experiment will be successful is the extent to which the OXPHOS complexes are extracted from the mitochondrial membranes. This in turn depends largely on the amount and type of detergent used. The optimal detergent should be able to extract the OXPHOS complexes sufficiently, but not denature the complexes. While the protocol we have described has been optimized for flight muscles (thoraces), modifications may be required for other tissues such as the brain (head extracts), gut, fat body and even larval muscles. This may require adjusting the detergent:protein ratio or even using other detergents such as n-Dodecyl-B-D-Maltoside (DDM). We also note that in-gel OXPHOS activity assays are semi-quantitative at best. Accordingly, for more quantitative results, additional mitochondrial assays such as using microplate readers to measure the specific activity of each of the OXPHOS enzymes spectrophotometrically should be used to confirm the in-gel activity results. Alternatively, mitochondrial respiration can be measured by high resolution respiratory units to validate any results obtained with the in-gel activity assays.

TROUBLESHOOTING

Problem 1

Gel electrophoresis takes too long to run (step 26).

Potential solution

Check the electrophoresis wire connections to ensure there are no loose connections to the power pack.

Problem 2

Protein streaks on the gel (step 33).

Potential solution

During step 14 collect only the supernatant and discard the pellet. If you see any particles in the sample (collected supernatant) re-centrifuge at high speed to collect only the supernatant.

Problem 3

Reduced or no in-gel activity (Complex I, II, IV and V) observed after electrophoresis (step 44 a- 44 d).

Potential solution

Ensure samples are processed at 4°C and perform the gel electrophoresis at 4°C. Processing the samples at room temperature and performing gel electrophoresis at room temperature causes protein denaturation and ultimately impairs activity of OXPHOS complexes.

Problem 4

Poorly resolved electrophoretic bands (step 33 & 40).

Potential solution

Add the appropriate amount of the blue dye to the protein sample during sample preparation.

Problem 5

Weak detection of higher molecular weight complexes in silver-stained gels (step 33).

Potential solution

Increase the duration of incorporation with the developer solution in step 32. However, while this makes the higher molecular weight complexes more visible, it comes at the expense of an increase in background staining in the vicinity of the lower molecular weight complexes.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Edward Owusu-Ansah (eo2364@cumc.columbia.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any new datasets or code.

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AUTHOR CONTRIBUTIONS

A. Murari and E. Owusu-Ansah worked together to organize and compose this protocol.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Deng, H., Dodson, M.W., Huang, H., and Guo, M. (2008). The Parkinson's disease genes pink1 and parkin promote mitochondrial fission and/or inhibit fusion in Drosophila. Proc. Natl. Acad. Sci. U S A. *105*, 14503–14508. https://doi.org/10.1073/pnas.0803998105.

Garcia, C.J., Khajeh, J., Coulanges, E., Chen, E.I., and Owusu-Ansah, E. (2017). Regulation of mitochondrial complex I biogenesis in Drosophila flight muscles. Cell Rep *20*, 264–278. https://doi.org/10.1016/j.celrep.2017.06.015.

Murari, A., Rhooms, S.K., Goparaju, N.S., Villanueva, M., and Owusu-Ansah, E. (2020). An antibody toolbox to track complex I assembly defines AIF's mitochondrial function. J. Cell Biol. *219*. https://doi.org/10.1083/jcb.202001071.

Owusu-Ansah, E., Song, W., and Perrimon, N. (2013). Muscle mitohormesis promotes longevity via systemic repression of insulin signaling. Cell *155*, 699–712. https://doi.org/10.1016/j.cell.2013.09.021.

Rana, A., Oliveira, M.P., Khamoui, A.V., Aparicio, R., Rera, M., Rossiter, H.B., and Walker, D.W. (2017). Promoting Drp1-mediated mitochondrial fission in midlife prolongs healthy lifespan of Drosophila melanogaster. Nat. Commun. *8*, 448. https://doi.org/10.1038/s41467-017-00525-4.

Ranganayakulu, G., Schulz, R.A., and Olson, E.N. (1996). Wingless signaling induces nautilus expression in the ventral mesoderm of the Drosophila embryo. Dev. Biol. *176*, 143–148. https://doi.org/10.1006/dbio.1996.9987.

Rera, M., Bahadorani, S., Cho, J., Koehler, C.L., Ulgherait, M., Hur, J.H., and Walker, D.W. (2011). Modulation of longevity and tissue homeostasis by the Drosophila PGC-1 homolog. Cell Metab. *14*, 623–634. https://doi.org/10.1016/j.cmet.2011.09.013.

Ulgherait, M., Rana, A., Rera, M., Graniel, J., and Walker, D.W. (2014). AMPK modulates tissue and organismal aging in a non-cell-autonomous manner. Cell Rep. *8*, 1767–1780. https://doi.org/10.1016/j.celrep.2014.08.006.