Blue native PAGE

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Blue native PAGE (BN-PAGE) can be used for one-step isolation of protein complexes from biological membranes and total cell and tissue homogenates. It can also be used to determine native protein masses and oligomeric states and to identify physiological protein–protein interactions. Native complexes are recovered from gels by electroelution or diffusion and are used for 2D crystallization and electron microscopy or analyzed by in-gel activity assays or by native electroblotting and immunodetection. In this protocol, we describe methodology to perform BN-PAGE followed by (i) native extraction or native electroblotting of separated proteins, or (ii) a second dimension of tricine-SDS-PAGE or modified BN-PAGE, or (iii) a second dimension of isoelectric focusing (IEF) followed by a third dimension of tricine-SDS-PAGE for the separation of subunits of complexes. These protocols for 2D and 3D PAGE can be completed in 2 and 3 days.

INTRODUCTION

BN-PAGE was developed for the separation of mitochondrial membrane proteins and complexes in the mass range of 10 kDa to 10 MDa[1–3], as exemplified in **Figure 1**. It is used for the one-step isolation of microgram amounts of membrane protein complexes from biological membranes[4–8] and total cell and tissue homogenates[9–13]; for clinical diagnostics of human mitochondrial disorders[10,11,13–15]; to determine native masses and oligomeric states[2,14–18]; to determine the stoichiometry of a multiprotein complex by an antibody-based gel-shift method[19]; to identify physiological protein–protein interactions[20–25]; for 2D crystallization and electron microscopy[26], in-gel activity assays[27–30], native electroblotting and immunodetection[15]; for studies of neurotransmitter assembly[31], protein import[32,33] and apoptosis research[9]; to isolate supramolecular physiological protein assemblies[15–25], and many more tasks. It is a convenient and inexpensive technique based on a few simple principles.

Nonionic detergents are used for solubilization of biological membranes. The choice of a specific nonionic detergent depends on the detergent stability of the protein complexes of interest. One of the mildest detergents is digitonin. It has been used to isolate supramolecular associations of multiprotein complexes, thus identifying physiological protein–protein interactions without using chemical crosslinking. Dodecylmaltoside has stronger delipidating properties compared to digitonin. It is a mild neutral detergent that is suited for isolation of membrane proteins and individual complexes but commonly dissociates labile hydrophobic interactions. Triton X-100 shows intermediate behavior with mitochondrial protein complexes. Under high-detergent conditions, it solubilizes membrane protein complexes, similar to dodecylmaltoside[21]. Under low-detergent conditions, it preserves respiratory supercomplexes[21] and dimeric ATP synthase[20], similar to digitonin.

Following solubilization of biological membranes and centrifugation, the anionic dye Coomassie blue G-250 is added to the supernatant. This dye is sufficiently soluble in water, but it can also bind to membrane proteins because of its hydrophobic properties. Binding a large number of dye molecules imposes a charge shift on the proteins that causes even basic proteins to migrate to the anode at pH 7.5 during BN-PAGE[2]. However, proteins are not separated according to the charge/mass ratio but according to size in acrylamide gradient gels. Protein migration gradually decelerates with running distance and with decreasing pore size of the gradient gel. Individual proteins must stop almost completely when they approach their size-dependent specific pore-size limit.

Because negatively charged protein surfaces repel each other, the tendency of membrane proteins to aggregate is considerably reduced. Furthermore, membrane surface areas lose their hydrophobic character upon binding the dye, which converts membrane proteins into water-soluble proteins. This means that no detergent is required in the BN gels once Coomassie dye has occupied protein surfaces. Therefore, the risk of denaturation of detergent-sensitive proteins is minimized during BN-PAGE.

Native proteins and complexes migrate as blue bands through BN gels. This facilitates excision of specific bands and recovery of blue stained native proteins by electroelution. The ease of recovery is especially advantageous when proteomic work that does not

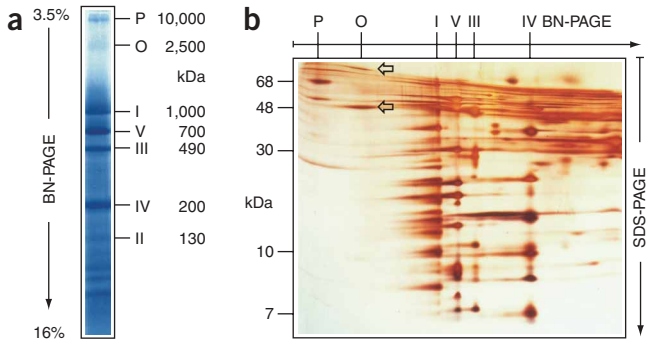

Figure 1 | Separation of dodecylmaltoside-solubilized mitochondrial complexes exemplifying the native mass range (<100 kDa to ~10 MDa) covered by BN-PAGE. (**a**) Solubilized bovine heart mitochondrial complexes I–V, pyruvate dehydrogenase complex (P), and oxoglutarate dehydrogenase complex (O) were separated according to their indicated masses on a linear 3.5→16% acrylamide gradient gel for BN-PAGE. (**b**) Subunits of the native complexes were separated by tricine-SDS-PAGE using a 13% T, 3% C gel type. Arrows mark the 96 kDa and 48 kDa subunits of the oxoglutarate dehydrogenase complex.

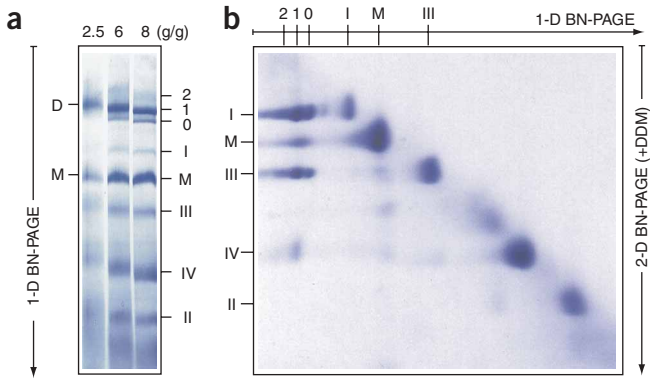

Figure 2 | Separation of supramolecular assemblies of oxidative phosphorylation complexes by 1D BN-PAGE and identification of their constituent individual complexes by 2D BN/BN-PAGE. (**a**) Bovine sonic particles were solubilized by digitonin using the indicated digitonin/protein ratios (g/g). The solubilized complexes were separated by 1D BN-PAGE. I, II, III and IV indicate mitochondrial complexes I–IV. 0, 1 and 2 indicate respiratory supercomplexes containing monomeric complex I, dimeric complex III, and zero (0), one (1) or two (2) copies of complex IV, respectively. M and D indicate monomeric (M) and dimeric (D) ATP synthase. (**b**) The lane for the digitonin/protein ratio of 8 (gram/gram or g/g) from **a** was processed by 2D BN/BN-PAGE using 0.02% dodecylmaltoside added to cathode buffer B (2D BN/BN-PAGE + DDM). Respiratory supercomplexes were thereby dissociated into the individual constituent complexes I, III and IV. 'g/g'.

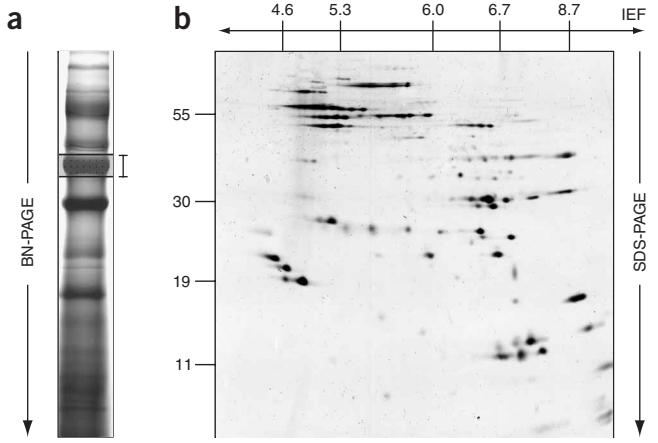

Figure 3 | Analysis of subunits of the mitochondrial ATP synthase complex from *Arabidopsis* by 3D BN/IEF/SDS-PAGE. (**a**) Mitochondrial protein complexes were first separated by BN-PAGE. Afterwards, a 600-kDa band (marked) representing the ATP synthase complex was cut out from the gel, electroeluted and separated from Coomassie blue. (**b**) Finally, the subunits of the ATP synthase complex were resolved by IEF/SDS-PAGE. The isoelectric points and molecular masses of standard proteins are given above and to the left of the gel. Reproduced from Werhahn and Braun[43] with permission.

demand homogeneously dissolved enzymes is to follow[34,35]. BN gels have also been used to isolate native proteins for 2D crystallization and electron microscopic analyses[26].

BN-PAGE is compatible with in-gel activity staining procedures[27–30] and in-gel detection of fluorescent labels like the CyDye fluorophors[36]. However, interference of Coomassie blue with activity measurements and fluorescent detection in general cannot be excluded. In these cases, a 'colorless native' (CN)-PAGE system might be used instead of BN-PAGE[6,37–39]. In its present form, the resolution capacity of CN-PAGE, with some exceptions, is low compared to BN-PAGE, and no suitable protocols for electroelution and electroblotting of proteins from CN gels are available so far. However, current investigations promise that the resolution of CN-PAGE can be improved considerably, and electroblotting of CN gels can work satisfactorily (I.W. and H.S., unpublished data).

Supramolecular assemblies that are retained during 1D BN-PAGE can be dissociated into the individual complexes by applying an orthogonal modified BN-PAGE for the second native dimension, thereby identifying the interaction partners and their stoichiometric ratio[6,21,23,29]. This second dimension of BN-PAGE, as exemplified in **Figure 2**, is less mild because low detergent amounts are added to the cathode buffer. The mixture of nonionic detergent and anionic Coomassie dye has some resemblance to an anionic detergent: it can dissociate detergent-labile associations but keeps individual respiratory complexes intact.

Protein subunits of complexes can be finally identified by various denaturing techniques: SDS-PAGE[40–42], doubled SDS-PAGE (dSDS-PAGE)[34] and IEF/SDS 3D PAGE[43], as exemplified in **Figure 3**.

MATERIALS

REAGENTS

- 1 M imidazole (Sigma); store at 7 °C. ▲ CRITICAL Use imidazole instead of the previously used Bis-Tris buffer, since Bis-Tris interferes with commonly used protein determination methods.
- 1 M imidazole/HCl, pH 7.0; store at 7 °C. ▲ CRITICAL Mark this stock solution with a color differing from that of the non–pH-adjusted stock above to avoid confusion.
- 20% dodecyl-β-D-maltoside (wt/vol) (Fluka), dissolved in water; store 1-ml aliquots at –20 °C
- 20% Triton X-100 (wt/vol) (Fluka), dissolved in water; store 1-ml aliquots at –20 °C
- 20% digitonin (wt/vol) (cat no. 37006, >50% purity, used without recrystallization) (Fluka), dissolved in water; store 0.1–1 ml aliquots at –20 °C. ▲ CRITICAL Heating (>70 °C) is required for some lots of digitonin, but avoid repeated freezing/thawing because this can lead to insolubility of digitonin at temperatures <50 °C.
- 1 M tricine (Fluka); store at 7 °C.
- Glycerol (Fluka)
- 2 M 6-aminohexanoic acid (Fluka); store at 7 °C.
- Acrylamide (twice-crystallized) (Serva)
- Bis-acrylamide (twice-crystallized) (Serva)
- 5% (wt/vol) stock of Coomassie blue G-250 (Serva) (suspend in 500 mM 6-aminohexanoic acid and store at 7 °C)
- Ponceau S/glycerol stock solution (0.1% Ponceau S, 50% glycerol, (wt/vol))
- 'Lysis' solution for IEF (optional): 8 M urea, 4% Triton X-100, 40 mM Tris-base, 50 mM DTT, 0.1 mM PMSF
- Methanol (for electroblotting of native proteins from BN gels)
- Acetic acid (for electroblotting of native proteins from BN gels)
- Mercaptoethanol (for tricine-SDS-PAGE)

EQUIPMENT

- Vertical electrophoresis apparatus according to Studier[44] without special cooling for BN and 2D SDS gels. Most commercially available vertical protein gel electrophoresis systems are also suitable for BN-PAGE, e.g., the SE 400 vertical unit (GE Healthcare) or the Protean II unit (Bio-Rad).
- Power supply (600 V, 500 mA) for BN-PAGE, 2D SDS-PAGE, and electroblotting of BN and SDS gels. For electroblotting, the power supply should have a minimal load resistance of 30 Ω or less.
- Semidry blotter with glassy carbon electrodes
- PVDF membranes (Immobilon P, Millipore)

TABLE 1 | Electrode and gel buffers for BN-PAGE.

	Cathode buffer B	Cathode buffer B/10	Anode buffer	Gel buffer (3×)
Tricine (mM)	50	50	–	–
Imidazole (mM)	7.5	7.5	25	75
Coomassie blue G-250 (%)	0.02	0.002	–	–
6-Aminohexanoic acid (M)	–	–	–	1.5
pH	~7.0[a]	~7.0[a]	7.0[b]	7.0[b]

Slightly blue cathode buffer B/10 and all other solutions except deeply blue cathode buffer B should be stored at 7 °C. 6-Aminohexanoic acid is not essential for BN-PAGE, but it is an efficient and inexpensive serine protease inhibitor. Using 500 mM concentration in gels prevents gel drying during prolonged storage. ▲ CRITICAL Deeply blue cathode buffer B should be stirred for several hours before use and stored at room temperature, since Coomassie dye can form aggregates at low temperature, which can prevent proteins from entering the gel. [a]No pH correction except with tricine or imidazole. [b]pH adjusted with HCl.

- Whatman chromatographic papers (17 CHR)
- Motor-driven tightly fitting 0.5–1 ml glass/Teflon Potter-Elvehjem homogenizer
- Peristaltic pump for casting acrylamide gradient gels
- Gradient mixer for use with a magnetic stirrer
- H-shaped elutor vessel (for electroelution of native proteins) can be built according to Hunkapiller *et al.*[45] or purchased from C.B.S. Scientific Co., Del Mar, CA.

REAGENT SETUP

Acrylamide-bisacrylamide mixture (AB-3) Prepare AB-3 mix containing 48 g acrylamide and 1.5 g bisacrylamide per 100 ml. Store at 7 °C. The AB-3 mix is defined as 49.5% T, 3% C according to Hjerten[46]. '% T' indicates the total concentration of acrylamide and bisacrylamide monomers. '% C' indicates the percentage of cross-linker (bisacrylamide) to total monomer. Unless otherwise stated, 3% C is used throughout. ! CAUTION Acrylamide and bisacrylamide are highly neurotoxic. When handling these chemicals, wear gloves and use a pipetting aid.

Electrode and gel buffers Prepare buffers as described in **Table 1**.

Solubilization buffers for BN-PAGE The solubilization buffers for BN-PAGE should be prepared as described in **Table 2**. Bis-Tris buffer was used in the original papers[1,2], but since Bis-Tris disturbs almost all protein determination assays, it is replaced here by imidazole buffer. Solubilization buffer A is commonly used to solubilize mitochondria and bacterial membranes. Higher salt concentrations should be avoided, since high salt can lead to considerable stacking of proteins in the sample wells of BN-PAGE, and highly concentrated membrane proteins tend to aggregate. If especially low ionic strength conditions are desired, 500 mM 6-aminohexanoic acid in buffer B can be used instead of 50 mM sodium chloride. Low concentrations of 6-aminohexanoic acid (2 mM) and EDTA (1 mM) are commonly added to all buffers for protease inhibition.

PROCEDURE

Selecting and casting gel ● TIMING ~2 h

1| Select and cast the appropriate gel type for BN-PAGE. Common gel types are acrylamide gradient gels, although, very rarely, non-gradient uniform acrylamide gels have been used for BN-PAGE[47].

▲ CRITICAL STEP Uniform acrylamide gels can be optimal for separation of two protein complexes with similar masses, but several attempts may be required to find the special acrylamide concentration for the specific narrow mass range.

First, cast gradient separation gels in the cold (4–7 °C) using a gradient mixer. The amount of each reagent to use to compose one gel (0.16 × 14 × 14 cm) is given in **Table 3**. Using a long cannula that reaches to the bottom of the gel, add 1 ml of water to the bottom, and then pump in the acrylamide solution beginning with the low acrylamide solution that is continuously underlain with solutions of increasing acrylamide concentration and density. Alternatively, if the gel-casting device includes a hole in the spacers between the two glass plates, the gradient gel also can be pumped from the bottom. Using a larger volume of the 4% acrylamide solution than of the 13% acrylamide solution containing glycerol assures that the two solutions initially are not mixed when the connecting tube is opened, and a linear gradient is obtained. 4→13% acrylamide gradient gels cover the protein mass range 10 kDa to 3 MDa. However, proteins smaller than 100 kDa are not well resolved. 3→13% acrylamide gradient gels extend the mass range up to 10 MDa. A 3% acrylamide sample gel is used for this 3→13% acrylamide gradient gel type.

2| Transfer the 'gel' to room temperature for polymerization. Polymerization takes approximately 30 min.

3| Remove water from the top of the gradient gel after polymerization and cast the sample gel at room temperature. Add the appropriate sample gel comb. Gels are used either as 'preparative gels' using the total 14-cm gel width for sample application or as 'analytical gels' containing 0.5-cm or 1.0-cm sample wells.

4| Leave the gel for approximately 15 min to polymerize.

5| Remove the sample gel comb and overlay the gel with gel buffer (1×).

■ PAUSE POINT The gel can be stored at 4 °C for 1 month if the overlay buffer is renewed at ~3-day intervals.

Isolation and storage of biological membranes

6| Membrane fractions of interest are isolated according to standard procedures.[22,37,48,49] Fractions may include total membranes of cells or membranes of subcellular fractions: e.g., organelles. Organelles often can be efficiently prepared by combining differential centrifugations and density centrifugations[22,37,48,49].

TABLE 2 | Solubilization buffers for BN-PAGE.

	Solubilization buffer A	Solubilization buffer B
Sodium chloride (mM)	50	–
Imidazole/HCl (mM)	50	50
6-Aminohexanoic acid (mM)	2	500
EDTA (mM)	1	1
pH (at 4°C)	7.0	7.0

TABLE 3 | Composition of a sample gel and 4% and 13% acrylamide mixtures to prepare an acrylamide gradient gel.

	Sample gel	Gradient separation gel	
	3.5% acrylamide	4% acrylamide	13% acrylamide
AB-3 mix	0.44 ml	1.5 ml	3.9 ml
Gel buffer 3x	2 ml	6 ml	5 ml
Glycerol	–	–	3 g
Water	3.4 ml	10.4 ml	3 ml
Total volume	6 ml	18 ml	15 ml
10% APS	50 µl	100 µl	75 µl
TEMED	5 µl	10 µl	7.5 µl

▲ CRITICAL 10% aqueous ammonium persulfate solution (APS) must be made up fresh or stored frozen. ▲ CRITICAL Use cold solutions (<7 °C) to avoid untimely polymerization in the tubings used.

7| Suspend biological membranes in buffers containing carbohydrate or glycerol (e.g., 250 mM sucrose, or 400 mM sorbitol, or 10% glycerol) to avoid dissociation of multiprotein complexes upon freezing.

8| Shock-freeze aliquots in liquid nitrogen and store at –80 °C. Ideally, the salt concentration in these membrane suspensions should be low (0–50 mM NaCl). Avoid potassium and divalent cations because these ions precipitate Coomassie dye and proteins together with the dye. It is preferable to use 50 mM imidazole/HCl, pH 7.0. Other buffers, e.g., sodium phosphate or sodium-MOPS, are tolerated at 5–20 mM concentrations.

■ **PAUSE POINT** Biological membranes can be stored at –80 °C for several months.

Solubilization of biological membranes ● **TIMING** ~**1 h**

9| Solubilize membranes as described in (A) for mitochondria and bacterial membranes, (B) for chloroplasts and (C) for mammalian cells and tissues.

(A) Solubilization of mitochondria and bacterial membranes for BN-PAGE

(i) Pellet aliquots of mitochondria (400 µg protein) by centrifuging for 10 min at 10,000–20,000*g*. Bacterial membrane fragments often require ultracentrifugation: e.g., 30 min at 100,000*g*. Centrifugation is required unless protein concentrations are larger than 40 mg ml^{-1}.

▲ CRITICAL STEP Dilute high-density samples several-fold with water to allow for sedimentation by centrifugation.

(ii) Add 40 µl solubilization buffer A (**Table 2**) to 400 µg of pelleted yeast mitochondria, mammalian mitochondria or bacterial membranes.

(iii) Homogenize the membranes by twirling a tiny spatula before adding detergent.

(iv) Add one of the detergents given in **Table 4**, using the volumes stated per 400 µg protein.

? TROUBLESHOOTING

(v) Leave sample to solubilize for 5–10 min.

(vi) Centrifuge for 15 min at 100,000*g*, or for 20 min at 20,000*g* if the sample contains multiprotein complexes of interest larger than 5 MDa. Retain the supernatant. In order to separate 5–10 MDa multiprotein complexes, use 3→13% or 3.5→13% acrylamide gradient gels for BN-PAGE.

(vii) Add 5 µl 50% glycerol to the supernatant.

(viii) Add Coomassie dye from the 5% Coomassie blue G-250 dye stock suspension to give a detergent/dye ratio of 8 (gram/gram): i.e., for yeast mitochondria, use 1 µl if you are using dodecylmaltoside, 2.4 µl if you are using Triton X-100 and 3 µl if you are using digitonin. For mammalian mitochondria use 2.5 µl if you are using dodecylmaltoside, 3 µl if you are using Triton X-100 and 6 µl if you are using digitonin. For bacterial membranes use 1 µl if you are using dodecylmaltoside, 2 µl if you are using Triton X-100 and 4 µl if you are using digitonin.

(ix) Load around 20 µl each to two 0.16 × 0.5 cm sample wells for BN-PAGE.

? TROUBLESHOOTING

TABLE 4 | Quantity of detergent required to solubilize the membranes.

	Yeast mitochondria	Mammalian mitochondria	Bacterial membranes
Dodecylmaltoside (20%)	2.0 µl (1.0 g/g)	5 µl (2.5 g/g)	2 µl (1.0 g/g)
Triton X-100 (20%)	4.8 µl (2.4 g/g)	6 µl (3.0 g/g)	4 µl (2.0 g/g)
Digitonin (20%)	6.0 µl (3.0 g/g)	12 µl (6.0 g/g)	8 µl (4.0 g/g)

The volumes of added detergent and the detergent/protein ratios chosen gram/gram; (indicated in brackets) are shown for solubilization of yeast mitochondria (*Saccharomyces cerevisiae* and *Yarrowia lipolytica*), mammalian mitochondria (bovine, human, rat, mouse, pig), and bacterial plasma membranes (*Paracoccus denitrificans*). Slightly modified protocols have been used for mitochondria of *Arabidopsis*, potato and other plants[23].

(B) Solubilization of chloroplasts for BN-PAGE

(i) Pellet chloroplasts (500 μg protein) by 10 min centrifugation at 20,000*g*.
(ii) Add 50 μl solubilization buffer A to the pelleted chloroplasts.
(iii) Homogenize the membranes by twirling a tiny spatula before adding detergent.
(iv) Add 2.5 μl 20% dodecylmaltoside or 20% Triton X-100 (to give a final concentration of 1.0 g/g) or 5 μl 20% digitonin (to give a final concentration of 2.0 g/g).
(v) Leave mixture to solubilize for 5–10 min.
(vi) Add 5 μl 50% glycerol.
(vii) Add 5% Coomassie dye to give a detergent/dye ratio of 8 (i.e., 1.25 μl if you are using dodecylmaltoside or Triton X-100 and 2.5 μl if you are using digitonin).
(viii) Load ~25 μl each to two 0.16 × 0.5 cm sample wells for BN-PAGE.

(C) Homogenization and solubilization of mammalian cells and tissues for BN-PAGE.

(i) Break up 10–50 mg tissue specimens with forceps and homogenize in 500 μl sucrose buffer (250 mM sucrose, 20 mM imidazole/HCl, pH 7.0) using a motor-driven, tightly fitting 0.5–1 ml glass/Teflon Potter-Elvehjem homogenizer (500 rpm; 10–20 strokes). Similarly, use 20–40 strokes (500 rpm) to homogenize 10–50 mg sedimented cells (e.g., mouse embryonic stems cells, human fibroblast and lymphoblast cell lines, 143B osteosarcoma and derived rho zero cell lines) in diluted sucrose buffer (83 mM sucrose, 6.6 mM imidazole/HCl, pH 7.0).
! CAUTION Wear protective gloves.
(ii) Divide homogenized samples into aliquots corresponding to 20 mg wet weight cells, 5 mg wet weight heart muscle, 15 mg wet weight skeletal muscle or 10 mg wet weight kidney, brain or liver, and centrifuge for 10 min at 10,000–20,000*g* to obtain a pellet containing nuclei, mitochondria and larger cell fragments.
■ **PAUSE POINT** Pellets can be stored at –80 °C following shock freezing by liquid nitrogen.
(iii) Add 35 μl solubilization buffer A to the pellet recovered from 20 mg wet weight cells, 5 mg wet weight heart muscle, 15 mg wet weight skeletal muscle or 10 mg wet weight kidney, brain or liver.
(iv) Homogenize by twirling a tiny spatula before adding detergent.
(v) Add dodecylmaltoside or digitonin. If using 20% dodecylmaltoside stock, add 5 μl for cells or skeletal muscle, 2 μl for heart muscle, or 10 μl for kidney, brain or liver. If using 20% digitonin stock, add 10 μl for cells or skeletal muscle, 4 μl for heart muscle, or 20 μl for kidney, brain or liver.
(vi) Solubilize for 5–10 min.
(vii) Centrifuge for 15 min at 100,000*g*. Retain the supernatant.
(viii) Add 5 μl 50% glycerol.
(ix) Add Coomassie dye from the 5% Coomassie blue G-250 dye stock suspension to give a detergent/dye ratio of 8: i.e., if you are using dodecylmaltoside add 2.5 μl to cells or skeletal muscle, 1 μl to heart muscle or 5 μl to kidney, brain and liver. If you are using digitonin add 5 μl to cells or skeletal muscle, 2 μl to heart muscle or 10 μl to kidney, brain or liver.
(x) Load ~20 μl each to two 0.16 × 0.5 cm sample wells for BN-PAGE.

Preparation of chromatographically prepurified proteins for BN-PAGE

10| Use samples with low ionic strength for separation by BN-PAGE (ideally in 50 mM NaCl, 50 mM imidazole/HCl, pH 7) to avoid protein aggregation in the sample gel during electrophoresis. Use dialysis or other desalting techniques (e.g., centrifuge filter units with appropriate cutoff limit, especially for high salt fractions from ion exchangers).
? TROUBLESHOOTING

11| Add 5% glycerol and 0.01% Ponceau S from the Ponceau S/glycerol stock solution but no Coomassie blue dye to chromatographically prepurified samples to avoid dissociation of detergent-labile subunits.
? TROUBLESHOOTING

Electrophoresis conditions ● TIMING 2–4 h

12| Perform BN-PAGE at 4–7 °C, since broadening of bands is observed at room temperature. Use cathode buffer B, and set power supply at 100 V until the sample has entered the gel.

13| Continue electrophoresis with the current limited to 15 mA and voltage limited to 500 V (for gel dimension of 0.16 × 14 × 14 cm).

14| After the blue running front has moved about one-third of the desired total running distance, remove cathode buffer B by suction pump (buffer B can be reused two or three times). Continue the run using slightly blue cathode buffer B/10 for better detection of faint protein bands, and to improve native blotting (less Coomassie dye competes under these conditions with protein binding to PVDF membranes).

15| Stop electrophoresis after 2–4 h. Use short runs if native extraction of proteins or 2D native electrophoresis is to follow. At that point the protein has not yet reached its pore size limit. Therefore it still has some mobility and can be efficiently extracted.

▲ **CRITICAL STEP** Coomassie dye diffuses, and faint protein bands that are just detected immediately after the end of electrophoresis may disappear after 1 d. Therefore, excise bands immediately following the electrophoresis run and store the excised bands in sealed tubes at 4 °C if electroelution or elution by diffusion is desired.

■ **PAUSE POINT** The blue gel or the excised bands can be stored at 4 °C for several days.

16| The BN gel can then be fixed and Coomassie and silver stained using a previously published protocol for staining of tricine-SDS gels[42]. The migration distances of individual bands relative to marker proteins can be used to estimate native masses of membrane proteins. Alternatively, using non-fixed BN gels, blue bands can be excised and electroeluted, as described in option (A); extracted by diffusion, as described in option (B); or electroblotted, as described in option (C). Elution by diffusion is an alternative to electroelution, especially suited for smaller gel volumes. Alternatively a second dimension of BN-PAGE can be performed, as described in option (D), a second dimension of tricine-SDS-PAGE, as described in (E); or a second dimension of IEF, as described in option (F). A third dimension of tricine-SDS-PAGE can be performed, as described in (E) and (F), after 2D BN/BN-PAGE or 2D BN/IEF-PAGE, respectively. For further techniques associated with tricine-SDS-PAGE, like staining and electroblotting, see tricine-SDS-PAGE in ref. 42.

? TROUBLESHOOTING

(A) Electroelution of native proteins ● **TIMING** 4–16 h (preferentially overnight)

(i) Excise blue protein bands from the BN gel (typically 0.3–0.5 ml volume per band from each preparative gel).

(ii) Mash the gel by squeezing it repeatedly from one syringe to the next.

(iii) Seal both lower ends of an H-shaped elutor vessel built according to Hunkapiller *et al.*[45] with low-cutoff (e.g., 2 kDa) dialysis membranes, because of their mechanical stability.

(iv) Squeeze the gel debris into the cathodic arm of the elutor vessel.

(v) Fill both arms of the H-shaped vessel and the connecting tube with electrode buffer (25 mM tricine, 3.75 mM imidazole, pH 7.0, and 5 mM 6-aminohexanoic acid for protease inhibition).

(vi) Extract proteins with voltage limited to 500 V for about 4 h.

! CAUTION Limit current to 2 mA per elutor vessel to prevent damage if a high-salt buffer was used erroneously. Extract proteins at 100 V overnight when protein aggregation must be avoided (e.g., for electron microscopic single-particle analysis).

(vii) Remove clear buffer using Pasteur pipettes with the glass tips covered by silicon tubing, and collect the dark blue protein/Coomassie layer.

(viii) Add 10% glycerol before shock-freezing and storing at –80 °C.

■ **PAUSE POINT** The blue sample containing glycerol can be stored at –80 °C for several months.

(B) Extraction by diffusion of native proteins ● **TIMING** 5–10 h

(i) Excise and mash blue protein bands as described above, or use a microhomogenizer for 1.5 ml cups. Extraction by diffusion is preferentially used for small gel volumes and has been used for a proteomic study[35].

(ii) Add water (ten times the gel volume), place the cup or sealed tube in a mixer, and let diffuse for 4 h or overnight.

(iii) Centrifuge for 10 min at 20,000*g* to pellet the gel debris, and proceed with the bluish supernatant.

(iv) Concentrate the solution by vacuum centrifugation (without heating) to approach the volume of the extracted gel piece. The concentrated solution then has a similar composition as the BN gel (500 mM 6-aminohexanoic acid, 25 mM imidazole/HCl, pH 7.0). If considerably higher protein concentrations are desired, e.g., for electron microscopic single-particle analysis, omit 6-aminohexanoic acid for casting the BN gels.

(C) Electroblotting of native proteins from BN gels ● **TIMING** 5 h

(i) Prepare electroblotting buffer (50 mM tricine, 7.5 mM imidazole; the resulting pH is around 7.0).

(ii) Wet PVDF membrane with methanol and transfer it to electroblotting buffer.

(iii) Soak a 3-mm stack of Whatman chromatographic papers with electroblotting buffer. The lower electrode is the cathode in this arrangement.

(iv) Place the gel and then the PVDF membrane on top.

(v) Soak another 3-mm stack of chromatographic papers with electroblotting buffer, put it on top, and mount the anode.

(vi) Place a 5-kg load on top.

(vii) Transfer for 3 h at 4 °C (preferred) or at room temperature. Set voltage to 20 V (actual voltage is around 7 V) and limit current to 0.5 mA cm^{-2} gel area.

(viii) Use 25% methanol, 10% acetic acid for background destaining; protein bands are often immediately visible after the transfer.

(ix) Rinse with water, dry and document the blot.

(x) For western blot analysis, destain the bands on the PVDF membrane completely with methanol (for about 3 min), and transfer then to western blot buffer.

? TROUBLESHOOTING

(D) Two-dimensional blue native PAGE (2D BN/BN-PAGE; native in two dimensions) ● **TIMING** 5–6 h

(i) Prepare buffers for the second dimension of modified BN-PAGE. Anode and gel buffers are the same as used for 1D BN-PAGE. Add 0.02% dodecylmaltoside or 0.03% Triton X-100 to cathode buffer B (see comment in D, step viii).

(ii) Excise a 0.5- to 1-cm-wide lane from the first dimension BN gel (gel thickness is ~1.6 mm), rinse the strip for several seconds with water, and place it on a glass plate at the usual position for sample gels.

(iii) Mount spacers (slightly thinner than for first-dimension gels: e.g., 1.4 mm), place the second glass plate on top, and fix with clamps.

(iv) Remove excess water and, after a few minutes, bring the gel to the upright position. (Waiting a few minutes avoids gliding of the gel strip.)

(v) Insert a long cannula through the gaps between the 1D gel strip and spacers, and cast a steep 4→16% acrylamide gradient gel (analogous to Step 1, **Table 3**) with some water on top of the gradient.

(vi) Following polymerization, add more water to cover the 1D native gel strip, and push the strip down to the separating gel using appropriate plastic cards.

(vii) Remove the water and fill the gaps between gel strip and spacers with a 10% acrylamide native gel (analogous to Step 1, **Table 3**).

(viii) Use cathode buffer B containing either 0.02% dodecylmaltoside or 0.03% Triton X-100, and start second-dimension BN-PAGE under the running conditions of first-dimension BN-PAGE (current limited to 15 mA; voltage increases gradually during the run from about 200 V to 500 V). In contrast to 1D BN gels, the 2D BN/BN gels should be stopped late in order to focus streaking protein bands finally into sharp protein spots. Cathode buffer B containing 0.02% dodecylmaltoside seems to dissociate physiological supramolecular structures more efficiently than cathode buffer B containing 0.03% Triton X-100.

(ix) There are several ways to proceed after 2D BN/BN-PAGE. Whole 2D BN/BN gels can be used for native blotting, as described in Step 16 (C); horizontal and vertical gel strips from 2D BN/BN-PAGE can be used for a third dimension of tricine-SDS-PAGE, as described in Step 16 (E), and blue protein spots from 2D BN/BN-PAGE can be extracted, e.g., by diffusion (Step 16 (B)) and then used for separation by IEF and SDS-PAGE, as described in Step 16 (F). Furthermore, 2D BN/BN PAGE is compatible with in-gel enzyme activity assays[24,30].

(E) Tricine-SDS-PAGE for resolution of subunits in the second or third dimension ● **TIMING** 3–6 h or overnight

(i) Place 0.5-cm gel strips from 1D BN-PAGE or 2D BN/BN-PAGE on glass plates.

(ii) Soak strips for 15 min with 1% SDS (or for 60–120 min with 1% SDS, 1% mercaptoethanol whenever cleavage of disulfide bridges is important).

(iii) Rinse briefly with water, especially when mercaptoethanol, an efficient inhibitor of polymerization, is used.

(iv) Mount spacers, which can be considerably thinner than the native gel (e.g., 0.7 mm for 1.4-mm native gels), and put the second glass plate on top. The 0.5-cm lanes of the native gel thereby are squeezed to a width of about 1 cm and will not move when the glass plates are brought to a vertical position.

(v) Pour the separating gel mixture between the glass plates, so that a 5-mm gap to the native gel strip is left, and overlay with water to fill the gap. Commonly a 10% T, 3% C tricine-SDS gel mixture is used to resolve proteins in the 5–100 kDa mass range and for efficient electroblotting. A 16% T, 3% C tricine-SDS gel mixture is used for the 5–70 kDa mass range and to obtain sharper bands compared to 10% gels. For experimental details, see recent tricine-SDS-PAGE protocols[41,42].

(vi) Following polymerization, gently push the native gel strip down to the separating gel using an 0.6-mm plastic card. Remove water and fill the gaps to the left and right of the native gel strip using a 10% acrylamide native gel mixture (analogous to Step 1, **Table 3**).

(vii) Start electrophoresis at room temperature, using an apparatus without special cooling and gels with dimensions 0.07 × 14 × 14 cm, with a maximal voltage of 200 V and current limited to 50 mA. When the current falls below 50 mA, the voltage can be increased with the current still limited to 50 mA. The run time for 10% and 16% gels is 3–4 h and 5–6 h, respectively.

? TROUBLESHOOTING

(F) Three-dimensional BN/IEF/SDS-PAGE ● **TIMING** ~20 h

(i) Electroelute proteins from BN gels as described above.

(ii) Precipitate proteins by adding a 10% (vol/vol) trichloroacetic acid (TCA) solution (final TCA concentration: 3.5%); incubate for 10 min at 0 °C and centrifuge at 13,000*g* for 10 min at 0 °C.

(iii) Resuspend proteins in acetone supplemented with 20 mM DTT, 1 mM PMSF; incubate for 1 h at –20 °C; and centrifuge at 13,000*g* for 15 min at 4 °C.

(iv) Repeat Step iii.

(v) Dry the pellet using a vacuum centrifuge.

(vi) Resuspend the pellet in 'lysis solution' for IEF (see REAGENTS).

(vii) Carry out isoelectric focusing (IEF) according to standard procedures. All basic strategies for IEF are suitable for the separation of proteins electroeluted from Blue native gels, like tube-gel separations using mobile ampholytes[50] or separations on gel strips using immobilized pH gradients[51].

(viii) Horizontally transfer the IEF gel strip onto an SDS polyacrylamide gel and carry out SDS-PAGE according to standard procedures[40,52]. Three-dimensional BN/IEF/SDS-PAGE allows separation of isoforms of subunits of protein complexes, which often have very similar molecular masses but differ with respect to their isoelectric points. Especially in mammals and higher plants, subunits of protein complexes are sometimes encoded by small protein families. For instance, the preprotein translocase of the outer mitochondrial membrane from *Arabidopsis* includes four isoforms for the pre-protein receptor TOM20, which can be separated nicely by 3D BN/IEF/SDS-PAGE[43].

● **TIMING**

Casting gel: 2 h
Sample preparation and loading: 1 h
1D blue native electrophoresis: 2–4 h, followed by fixing (0.5 h) and Coomassie staining (1 h) or the following optional steps:
Step 16 (A) electroelution: 4–16 h (preferentially overnight)
Step 16 (B) recovery by diffusion/concentration: 5–10 h
Step 16 (C) native blotting: 5 h
Step 16 (D) 2D BN/BN-PAGE (native in two dimensions): 5–6 h, followed (optionally) by 3D SDS-PAGE (3–6 h or overnight), fixing (0.5 h) and Coomassie staining (1 h)
Step 16 (E) 2D tricine-SDS-PAGE: 3–6 h or overnight followed by fixing (0.5 h) and Coomassie staining (1 h)
Step 16 (F) IEF: 16 h, followed by tricine-SDS-PAGE (3–6 h or overnight), fixing (0.5 h) and Coomassie staining (1 h)

TROUBLESHOOTING

Troubleshooting advice can be found in **Table 5**.

TABLE 5 | Troubleshooting table.

PROBLEM	SOLUTION
Step 9 (A) How to optimize membrane solubilization and to identify potential detergent sensitivity of membrane protein complexes.	For initial BN analyses of specific biological membranes, test at least three detergent/protein ratios: a normal detergent/protein ratio as given in Step 9 (A), **Table 4**, a second one with half the detergent, and a third one with doubled the detergent. This helps to find the optimal detergent amount for membrane solubilization and may indicate whether a specific enzyme is detergent sensitive with respect to the physiological state of association and the catalytic activity. For example, yeast ATP synthase might be dimeric using low Triton X-100 but monomeric using high Triton X-100. Monomeric and dimeric ATP synthase differ by three dimer-specific subunits, which can be analyzed by second-dimension SDS-PAGE. Another possibility to preserve physiological oligomeric states is to use digitonin instead of Triton X-100 or dodecylmaltoside. Digitonin seems to be less delipidating, and the digitonin/protein ratio chosen is less critical.
Step 9 (A) Upper and lower loading limits.	Using isolated mitochondria, the maximum protein load for 0.16 × 1.0 cm sample wells is around 400 μg of total protein. Blue aggregates that can prevent proteins from entering the gel can appear in the sample gel during electrophoresis if the load is too high. In that situation, keep the solubilization conditions as described but apply half the sample volumes. The minimal load to BN gels depends on the sensitivity of the protein detection method. Be sure that the detergent/protein ratios are chosen as described for solubilization of normal protein amounts, and use low final volumes for low protein amounts in order to keep detergent concentrations clearly above the critical micelle concentration (cmc).
Step 10 Protein stacking and aggregation. Stacking of proteins (concentration to sharp bands in the sample gel) increases with the salt concentration in the sample. Unfortunately, membrane protein complexes tend to aggregate upon concentration.	For this reason, and because high salt precipitates Coomassie dye, salt concentrations must be kept low. Lithium and sodium salts should be used preferentially to avoid precipitation of Coomassie dye and Coomassie–protein complexes by potassium and divalent cations.

TABLE 5 | Troubleshooting table (continued).

Step 11 Dissociating properties of detergent/dye mixtures. Chromatographically prepurified membrane proteins often contain reduced amounts of boundary lipid and excess detergent. Mixing excess detergent with added Coomassie dye generates anionic detergent-like properties and dissociates detergent-labile subunits from multiprotein complexes.	Do not add Coomassie dye to chromatographically pre-purified membrane proteins before application to BN-PAGE. Coomassie dye from the cathode buffer that reaches sample proteins later during BN-PAGE seems to cause less dissociation. This might be explained by preseparation of acidic proteins from the bulk of neutral detergent during electrophoresis while, at the same time, Coomassie dye is retarded by incorporation into detergent/lipid micelles in the sample well. After saturation with dye, these mixed micelles may catch up with the protein in the separating gel where it is no longer exposed to excess detergent. Preseparation is restricted to acidic proteins (intrinsic pI < 7) that migrate to the anode (in the absence of Coomassie dye) while the bulk of neutral detergent stays immobile in the sample well until it becomes charged by Coomassie dye.
Step 16 Problems with determination of native masses by BN-PAGE. BN-PAGE can be used for rapid mass estimation of native membrane proteins and complexes and, with some limitations, for water-soluble proteins[2]. The error commonly is in the 10–20% range, but larger deviations have also been reported, especially with highly basic membrane proteins, since large numbers of basic amino acid residues often cannot be sufficiently overcome by negative charges of protein bound Coomassie dye molecules[2,19,54,55]. In such cases (i.e., in the absence of a clear excess of negative charges) a shift to higher apparent protein masses is observed. Apparent deviations from calculated masses (summing up the masses of subunits) may also result from glycosylation[7]. Some deviations, however, cannot be assigned to the above mentioned protein specific problems but rather to inappropriate use of BN-PAGE.	As a general rule for mass determination by BN-PAGE, use the same detergent for solubilization of your membrane protein of interest and for the membrane proteins used for mass calibration (e.g., the well-characterized mitochondrial oxidative phosphorylation complexes), since membrane protein migration distances can vary considerably with the detergent used. This variation presumably correlates with variable detergent-dependent amounts of boundary lipid. In the original work on mass determination by BN-PAGE[2], some water-soluble proteins were identified that can substitute for membrane protein markers; i.e., these water-soluble proteins matched the calibration curve of dodecylmaltoside-solubilized membrane protein complexes. Since the migration distances of dodecylmaltoside-solubilized and digitonin-solubilized membrane protein complexes differ considerably, it is not possible to use the same set of water-soluble proteins to estimate the native masses of digitonin-solublized membrane proteins. Ignoring this fact can lead to considerable errors.
Step 16 (C) Inefficient transfer of proteins by native electroblotting.	Similar to native electroelution and extraction, stop BN-PAGE early for native electroblotting to ensure efficient transfer of proteins. Do not use cathode buffer B as the final buffer before electroblotting; rather, use B/10, since Coomassie dye binds to PVDF membranes and thereby reduces protein binding capacity. Do not use nitrocellulose membranes, since these membranes cannot be destained under the conditions described above.[3]
Step 16 (C) Electroblotting conditions may depend on the specific apparatus used.	Limit current to 0.5 mA cm^{-2} gel area. The actual voltage may vary with the apparatus used (and the thickness of the paper stacks).
Step 16 (E) Choice of electrophoresis system. Although the Laemmli Tris-glycine system[52] exhibits excellent resolution in 1D SDS-PAGE, it is not advisable to use it for the special type of 2D or 3D electrophoresis discussed here. Protein bands often streak, and the electrotransfer of hydrophobic proteins to PVDF membranes is problematic, because a higher-percentage acrylamide concentration must be used for glycine SDS-PAGE than for tricine-SDS-PAGE to cover the same separation range.	Use tricine-SDS gels for the 2D and 3D electrophoresis types described here.
Step 16 (E) In some situations, SDS-PAGE may not provide sufficient resolution (e.g., for mass spectrometric identification of hydrophobic proteins in a mixture of common proteins).	In this case, use doubled SDS-PAGE (dSDS-PAGE)[34], a technique that uses two orthogonal SDS-PAGEs with strongly differing gel types for 1D and 2D separation. Similarly, dSDS-PAGE using nonreducing conditions for 1D resolution and reducing conditions for 2D resolution is ideal for identifying the protein interaction partners after chemical crosslinking by crosslinkers containing cleavable disulfide bonds.

ANTICIPATED RESULTS

Blue native PAGE, similar to coimmunoprecipitation, has the advantage that detergent must be added only once (for solubilization), and this detergent generally is not harmful to the proteins, owing to the presence of lipid from the solubilized membranes. Unlike coimmunoprecipitation, BN-PAGE does not require antibodies to isolate the desired protein but seems to be less mild because of the anionic properties of added Coomassie dye.

BN-PAGE has mostly been used for protein separation on the analytical (microgram) scale. It covers a mass range of native proteins and complexes from <100 kDa to ~ 10 MDa, as exemplified in **Figure 1**. On the preparative (up to milligram) scale, it has been used, e.g., for Edman degradation of the subunits of oxidative phosphorylation complexes[53]. Using biological membranes with highly abundant protein complexes of interest, it is often possible to identify protein complexes as blue bands during 1D BN-PAGE and to identify subunits by Coomassie staining following 2D SDS-PAGE. Using sources with less-abundant proteins of interest, one might have to use silver staining, immunodetection or detection of covalently linked CyDye fluorophores to identify the proteins of interest. However, this is no great problem and essentially doesn't involve different handling, with the exception of attempting to enrich the desired protein before solubilization for BN-PAGE.

In recent years, BN-PAGE has also been used to identify and isolate larger physiological protein assemblies: e.g., supramolecular associations of ATP synthase and respiratory chain supercomplexes, as exemplified in **Figure 2**. The identity of the constituent complexes is relatively easy to determine by 2D BN/BN-PAGE (**Fig. 2b**); i.e., by a 2D system that is native in both dimensions. The supercomplexes separated in the first-dimension gel are dissociated into the individual complexes in the second-dimension gel, which is operated under less mild conditions than 1D BN-PAGE.

In parallel with the progress in analytical and mass spectrometric techniques, the isolation protocols can be scaled down or combined to multidimensional techniques, as exemplified in **Figure 3**, which shows the analysis of a multisubunit complex by 3D (BN/IEF/SDS) PAGE. This combination is quite a powerful approach for characterizing different isoforms of subunits of protein complexes that would not be accessible by common SDS-PAGE.

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1. Schägger, H. & von Jagow, G. Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal. Biochem.* **199**, 223–231 (1991).
2. Schägger, H., Cramer, W.A. & von Jagow, G. Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. *Anal. Biochem.* **217**, 220–230 (1994).
3. Schägger, H. Blue native electrophoresis, in *Membrane Protein Purification and Crystallization. A Practical Guide* 2nd edn. (eds. Hunte, C., von Jagow, G. & Schägger, H.) 5.105–5.130 (Academic, San Diego, USA, 2003).
4. Kügler, M., Jänsch, L., Kruft, V., Schmitz, U.K. & Braun, H.P. Analysis of the chloroplast protein complexes by blue-native polyacrylamide gelelectrophoresis. *Photosynth. Res.* **53**, 35–44 (1997).
5. Schägger, H. Blue native gels to isolate protein complexes from mitochondria. *Methods Cell Biol.* **65**, 231–244 (2001).
6. Pfeiffer, K. *et al.* Cardiolipin stabilizes respiratory chain supercomplexes. *J. Biol. Chem.* **278**, 52873–52880 (2003).
7. Ludwig, J. *et al.* Identification and characterization of a novel 9.2 kDa membrane sector associated protein of vacuolar proton-ATPase from chromaffin granules. *J. Biol. Chem.* **273**, 10939–10947 (1998).
8. Schägger, H. Native electrophoresis for isolation of mitochondrial oxidative phosphorylation protein complexes. *Methods Enzymol.* **260**, 190–202 (1995).
9. Vahsen, N. *et al.* AIF deficiency compromises oxidative phosphorylation. *EMBO J.* **23**, 4679–4689 (2004).
10. Acin-Perez, R. *et al.* Respiratory complex III is required to maintain complex I in mammalian mitochondria. *Mol. Cell* **13**, 805–815 (2004).
11. Schägger, H. *et al.* Significance of respirasomes for the assembly/stability of human respiratory chain complex I. *J. Biol. Chem.* **279**, 36349–36353 (2004).
12. Camacho-Carvajal, M., Wollscheid, B., Aebersold, R., Steimle, V. & Schamel, W.A. Two-dimensional blue native/SDS gel electrophoresis of multi-protein complexes from whole cellular lysates. *Mol. Cell. Proteomics* **3**, 176–182 (2004).
13. Schägger, H. *et al.* Electrophoretic separation of multiprotein complexes from blood platelets and cell lines: Technique for the analysis of diseases with defects in oxidative phosphorylation. *Electrophoresis* **17**, 709–714 (1996).
14. Schägger, H. Quantification of oxidative phosphorylation enzymes after blue native electrophoresis and two-dimensional resolution: Normal complex I protein amounts in Parkinsońs disease conflict with reduced catalytic activities. *Electrophoresis* **16**, 763–770 (1995).
15. Carrozzo, R. *et al.* Subcomplexes of human ATP synthase mark mitochondrial biosynthesis disorders. *Ann. Neurol.* **59**, 265–275 (2006).
16. Dudkina, N.V., Heinemeyer, J., Keegstra, W., Boekema, E.J. & Braun, H.P. Structure of dimeric ATP synthase from mitochondria: An angular association of monomers induces the strong curvature of the inner membrane. *FEBS Lett.* **579**, 5769–5772 (2005).
17. Dudkina, N.V., Eubel, H., Keegstra, W., Boekema, E.J. & Braun, H.P. Structure of a mitochondrial supercomplex formed by respiratory-chain complexes I and III. *Proc. Natl. Acad. Sci. USA* **102**, 3225–3229 (2005).
18. Minauro-Sanmiguel, F., Wilkens, S. & Garcia, J.J. Structure of dimeric mitochondrial ATP synthase: novel F_0 bridging features and the structural basis of mitochondrial cristae biogenesis. *Proc. Natl. Acad. Sci. USA* **102**, 12356–12358 (2005).
19. Schamel, W.W. *et al.* Coexistence of multivalent and monovalent TCRs explains high sensitivity and wide range of response. *J. Exp. Med.* **202**, 493–503 (2005).
20. Arnold, I., Pfeiffer, K., Neupert, W., Stuart, R.A. & Schägger, H. Yeast mitochondrial F_1F_0-ATP synthase exists as a dimer: identification of three dimer-specific subunits. *EMBO J.* **17**, 7170–7178 (1998).
21. Schägger, H. & Pfeiffer, K. Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. *EMBO J.* **19**, 1777–1783 (2000).
22. Stroh, A. *et al.* Assembly of respiratory chain complexes I, III, and IV into NADH oxidase supercomplex stabilizes Complex I in *Paracoccus denitrificans*. *J. Biol. Chem.* **279**, 5000–5007 (2004).
23. Eubel, H., Jänsch, L. & Braun, H.P. New insights into the respiratory chain of plant mitochondria: supercomplexes and a unique composition of complex II. *Plant Physiol.* **133**, 274–286 (2003).
24. Eubel, H., Heinemeyer, J. & Braun, H.P. Identification and characterization of respirasomes in potato mitochondria. *Plant Physiol.* **134**, 1450–1459 (2004).
25. Paumard, P. *et al.* The ATP synthase is involved in generating mitochondrial cristae morphology. *EMBO J.* **21**, 221–230 (2002).
26. Poetsch, A., Neff, D., Seelert, H., Schägger, H. & Dencher, N.A. Dye removal, catalytic activity and 2D-crystallization of chloroplast H^+-ATP synthase purified by blue native electrophoresis. *Biochim. Biophys. Acta* **1466**, 339–349 (2000).
27. Zerbetto, E., Vergani, L. & Dabbeni-Sala, F. Quantification of muscle mitochondrial oxidative phosphorylation enzymes via histochemical

staining of blue native polyacrylamide gels. *Electrophoresis* **18**, 2059–2064 (1997).
28. Jung, C., Higgins, C.M.J. & Xu, Z. Measuring the quantity of mitochondrial electron transport chain complexes in tissues of central nervous system using Blue native polyacrylamide gel electrophoresis. *Anal. Biochem.* **286**, 214–223 (2000).
29. Eubel, H., Heinemeyer, J., Sunderhaus, S. & Braun, H.P. Respiratory chain supercomplexes in plant mitochondria. *Plant Physiol. Biochem.* **42**, 937–942 (2004).
30. Sunderhaus, S. *et al.* Carbonic anhydrase subunits form a matrix-exposed domain attached to the membrane arm of mitochondrial complex I in plants. *J. Biol. Chem.* **281**, 6482–6488 (2006).
31. Griffon, N. *et al.* Molecular determinants of glycine receptor subunit assembly. *EMBO J.* **18**, 4711–4721 (1999).
32. Dietmeyer, K. *et al.* Tom 5 functionally links mitochondrial preprotein receptors to the general import pore. *Nature* **388**, 195–200 (1997).
33. Jänsch, L., Kruft, V., Schmitz, U.K. & Braun, H.P. Unique composition of the preprotein translocase of the outer mitochondrial membrane from plants. *J. Biol. Chem.* **273**, 17251–17257 (1998).
34. Rais, I., Karas, M. & Schägger, H. Two-dimensional electrophoresis for the isolation of integral membrane proteins and mass spectrometric identification. *Proteomics* **4**, 2567–2571 (2004).
35. Fandino, A.S. *et al.* LC-nanospray-MS/MS analysis of hydrophobic proteins from membrane protein complexes isolated by blue-native electrophoresis. *J. Mass Spectrom.* **40**, 1223–1231 (2005).
36. Perales, M. *et al.* Disruption of a nuclear gene encoding a mitochondrial gamma carbonic anhydrase reduces complex I and supercomplex I+III2 levels and alters mitochondrial physiology in *Arabidopsis*. *J. Mol. Biol.* **350**, 263–277 (2005).
37. Wittig, I. & Schägger, H. Advantages and limitations of clear native polyacrylamide gel electrophoresis. *Proteomics* **5**, 4338–4346 (2005).
38. Gavin, P.D., Devenish, R.J. & Prescott, M. FRET reveals changes in the F_1-stator stalk interaction during activity of F_1F_0-ATP synthase. *Biochim. Biophys. Acta* **1607**, 167–179 (2003).
39. Gavin, P.D., Prescott, M. & Devenish, R.J. Yeast F_1F_0-ATP synthase complex interactions in vivo can occur in the absence of the dimer specific subunit e. *J. Bioenerg. Biomembr.* **37**, 55–66 (2005).
40. Schägger, H. & von Jagow, G. Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1–100 kDalton. *Anal. Biochem.* **166**, 368–379 (1987).
41. Schägger, H. SDS electrophoresis techniques, in *Membrane Protein Purification and Crystallization. A Practical Guide* 2nd edn. (eds. Hunte, C., von Jagow, G. & Schägger, H.) 4.85–4.103 (Academic, San Diego, USA, 2003).
42. Schägger, H. Tricine–SDS-PAGE. *Nat. Protocols* **1**, 16–22 (2006).
43. Werhahn, W. & Braun, H.P. Biochemical dissection of the mitochondrial proteome from *Arabidopsis thaliana* by three-dimensional gel electrophoresis. *Electrophoresis* **23**, 640–646 (2002).
44. Studier, F.W. Analysis of bacteriophage T7 early RNAs and proteins on slab gels. *J. Mol. Biol.* **79**, 237–248 (1973).
45. Hunkapiller, M.W., Lujan, E., Ostrander, F. & Hood, L.E. Isolation of microgram quantities of proteins from polyacrylamide gels for amino acid sequence analysis. *Methods Enzymol.* **91**, 227–236 (1983).
46. Hjerten, S. Chromatographic separation according to size of macromolecules and cell particles on columns of agarose suspensions. *Arch. Biochem. Biophys.* **99**, 466–475 (1962).
47. Reif, S., Voos, W. & Rassow, J. Intramitochondrial dimerization of citrate synthase characterized by blue native electrophoresis. *Anal. Biochem.* **288**, 97–99 (2001).
48. Meisinger, C., Pfanner, N. & Truscott, K.N. Isolation of yeast mitochondria. *Methods Mol. Biol.* **313**, 33–39 (2006).
49. Heinemeyer, J., Eubel, H., Wehmhöner, D., Jänsch, L. & Braun, H.P. Proteomic approach to characterize the supramolecular organization of photosystems in higher plants. *Phytochemistry* **65**, 1683–1692 (2004).
50. Klose, J. & Kobalz, U. Two-dimensional electrophoresis of proteins: an updated protocol and implications for a functional analysis of the genome. *Electrophoresis* **16**, 1034–1059 (1995).
51. Görg, A. & Weiss, W., High-resolution two-dimensional electrophoresis of proteins using immobilized pH gradients. in *Cell Biology. A Laboratory Handbook* (ed. Celis, J.) 386–397 (Academic, New York, 1998).
52. Laemmli, U.K. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680–685 (1970).
53. Schägger, H., Brandt, U., Gencic, S. & von Jagow, G. Ubiquinol-cytochrome c-reductase from human and bovine mitochondria. *Methods Enzymol.* **260**, 82–96 (1995).
54. Moro, F., Sirrenberg, C., Schneider, H.C., Neupert, W. & Brunner, M. The TIM17.23 preprotein translocase of mitochondria: composition and function in protein transport into the matrix. *EMBO J.* **18**, 3667–3675 (1999).
55. Schamel, W.W. & Reth, M. Monomeric and oligomeric complexes of the B cell antigen receptor. *Immunity* **13**, 5–14 (2000).