

# Chapter 2

## Functional Assembly of Soluble and Membrane Recombinant Proteins of Mammalian NADPH Oxidase Complex

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### Abstract

Activation of phagocyte cells from an innate immune system is associated with a massive consumption of molecular oxygen to generate highly reactive oxygen species (ROS) as microbial weapons. This is achieved by a multiprotein complex, the so-called NADPH oxidase. The activity of phagocyte NADPH oxidase relies on an assembly of more than five proteins, among them the membrane heterodimer named flavocytochrome  $b_{558}$  (Cyt $b_{558}$ ), constituted by the tight association of the gp91<sup>phox</sup> (also named Nox2) and p22<sup>phox</sup> proteins. The Cyt $b_{558}$  is the membrane catalytic core of the NADPH oxidase complex, through which the reducing equivalent provided by NADPH is transferred via the associated prosthetic groups (one flavin and two hemes) to reduce dioxygen into superoxide anion. The other major proteins (p47<sup>phox</sup>, p67<sup>phox</sup>, p40<sup>phox</sup>, Rac) requisite for the complex activity are cytosolic proteins. Thus, the NADPH oxidase functioning relies on a synergic multi-partner assembly that in vivo can be hardly studied at the molecular level due to the cell complexity. Thus, a cell-free assay method has been developed to study the NADPH oxidase activity that allows measuring and eventually quantifying the ROS generation based on optical techniques following reduction of cytochrome c. This setup is a valuable tool for the identification of protein interactions, of crucial components and additives for a functional enzyme. Recently, this method was improved by the engineering and the production of a complete recombinant NADPH oxidase complex using the combination of purified proteins expressed in bacterial and yeast host cells. The reconstitution into artificial membrane leads to a fully controllable system that permits fine functional studies.

**Key words** Cell-free assays, NADPH oxidase, Nox, Superoxide anion, Cytochrome  $b_{558}$ , ferricytochrome c, gp91<sup>phox</sup>, Membrane protein complex, Recombinant protein, *Pichia pastoris*, Arachidonic acid, Liposomes

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## 1 Introduction

The NADPH oxidase complex is well known as the source for the massive superoxide radical production in phagocytes. It is formed from the assembly of at least five cytosolic and membrane proteins which produces superoxide from the oxidation of NADPH. The key membrane-associated component of the NADPH oxidase is the flavocytochrome  $b_{558}$  heterodimer composed by a 91-kDa

glycoprotein, gp91<sup>phox</sup> (also called Nox2) and a 22-kDa protein (p22<sup>phox</sup>). Exposure to microorganisms or inflammatory mediators activates these cells and through multiple signaling pathways, cytosolic subunits (p67<sup>phox</sup>, p47<sup>phox</sup>, small G protein Rac1/2, and p40<sup>phox</sup>) translocate to the membrane from cytosol and form the functional complex. The functioning of the NADPH oxidase complex in vivo is difficult to decipher due to the complexity of cell signaling pathways and the impossibility of controlling the presence of the partners in function of time. Although the comparison of results obtained in vitro and in vivo may not be straightforward, the alternative way was to develop the so-called cell-free assay that enables in vitro studies of the events occurring during NADPH oxidase activation and activity giving detailed information on each step of assembly and on the reaction that could not be easily observed in vivo. The method of cell-free activation of NADPH oxidase was discovered in Pick's laboratory and was first described in 1984. This approach consists of purified membranes containing native Cytb<sub>558</sub> (isolated from bovine or human neutrophils) to which the cytosolic fraction (from neutrophils) is added in the presence of suitable additives. This first system evolved nowadays into mainly three cell-free assay systems in which the major difference resides in the nature of the membrane component: (1) the *semi-recombinant cell-free assay*: the most frequent "canonical" cell-free system in which the membrane components are the purified membranes isolated from bovine or human neutrophils but the cytosol fraction is replaced by purified cytosolic proteins (for review see [1]); (2) the recombinant cell-free system in which the neutrophil membranes are replaced by yeast (*Pichia pastoris*) membranes containing the recombinant rCytb<sub>558</sub>; (3) the artificial cell-free assay in which the recombinant or native Cytb<sub>558</sub> is reconstituted into lipid vesicles. In all cases, the cytosolic proteins are routinely produced in *E. coli*, purified [2] and added as desired to permit a strict quantification of each protein. NADPH is added and the measurement of the generated superoxide anions rate by the assembled NADPH oxidase complex is followed by SOD-inhibited cytochrome c reduction (see **Note 1**).

The bottleneck to produce functional recombinant rCytb<sub>558</sub> (gp91<sup>phox</sup>/p22<sup>phox</sup>) was overcome by using the methylotrophic yeast *Pichia pastoris* expression system that has been shown to be a valuable tool to produce the recombinant bovine heterodimer [3]. The *Pichia pastoris* expression system is capable of making the major eukaryotic posttranslational modifications in the overexpression of heterologous proteins. The maturation (glycosylation form of gp91<sup>phox</sup>), the heterodimer formation, and the prosthetic group association to the recombinant proteins produced in yeast membranes have been confirmed. Since then, many yeast clones were generated by using different vector constructions that express the heterodimer rCytb<sub>558</sub> with different tag positions and also the gp91<sup>phox</sup> protein as a monomer.

The flexibility brought by the molecular biology combined to yeast expression system and the cell-free assays allowed investigations of the functioning of improbable component assembly. It has been shown, in particular, the capacity of gp91<sup>phox</sup> to produce superoxide in the absence of its membrane partner p22<sup>phox</sup> [4]. Collected information on the maturation and the activity of the recombinant gp91<sup>phox</sup> in the absence of p22<sup>phox</sup> allowed proposing a role of p22<sup>phox</sup> in the complex stabilization, its absence leading to an unconventional way of NADPH oxidase assembly. The protocols to obtain the “routine” semi-recombinant cell-free assay system have been described previously in detail (*see* in [5] and updated in [6]). In this chapter, we will focus on the methods to elaborate the “entirely recombinant” cell-free assay system which mix recombinant cytosolic proteins (produced in *E. coli*) with recombinant membrane oxidase proteins (produced in yeast) embedded into the host yeast membranes (plasma or subcellular organelle membranes from *P. pastoris*) or into artificial membranes.

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## 2 Materials

### 2.1 Cells, Media, and Buffers

1. Restriction enzymes and other DNA-modifying enzymes (New England Biolabs, United Kingdom).
2. Reagents for DNA extraction and purification were from VWR International LLC (USA) and primers (Eurogentec, Angers, France).
3. *Pichia pastoris* strains X33 and SMD1168 and pAO815 and pPICZ $\alpha$ A expression vectors (Invitrogen, Grand Island, NY, USA).
4. *NEB5 $\alpha$*  bacterial strain for plasmid preparation and cloning (New England Biolabs, United Kingdom).
5. The rich medium YPD for growing yeast under nonselective conditions [1% (w/v) yeast extract, 2% (w/v) peptone, 2% dextrose and 2% agar if making solid medium].
6. 10 $\times$  YNB (13.4% Yeast Nitrogen Base with Ammonium Sulfate without amino acids): solubilize 34 g of YNB without ammonium sulfate and amino acids and 100 g of ammonium sulfate in 1000 mL of water and filter sterilize. Heat the solution to dissolve YNB completely. Store at 4 °C.
7. BMGY and BMMY (Buffered Glycerol-complex Medium, Buffered Methanol-complex Medium): dissolve 10 g of yeast extract, 20 g peptone in 700 mL water. Autoclave 20 min. Cool to room temperature, add the following and mix well: 100 mL 1 M potassium phosphate buffer, pH 6.0, 100 mL 10 $\times$  YNB, 2 mL 0.02% biotin, 100 mL 20% glycerol. For BMMY, add 10 mL 100% methanol (added just before use) instead of glycerol. Store media at 4 °C.

## **2.2 For Heterologous Expression of Recombinant Membrane Proteins in *Pichia pastoris* Cells**

1. *P. pastoris* X33 and SMD1168 host strain colonies grown on YPD agar plates (max. 2–3 days old).
2. *Pichia* EasyComp transformation kit from Invitrogen (Grand Island, NY, USA).
3. Shaking incubator (250 rpm).
4. 10× Dextrose solution: Dissolve 200 g D-glucose into 1 L water and filter sterilize on a 0.2 µm membrane.
5. YPD medium: dissolve 10 g yeast extract and 20 g peptone into 900 mL water.
6. 10× YNB: dissolve 134 g yeast nitrogen base (YNB) with ammonium sulfate (100 g/L) w/o amino acids into 1 L water and filter sterilize.
7. 500× Biotin solution: dissolve 20 mg biotin in 100 mL water, filter sterilized.
8. Breaking buffer: 50 mM sodium phosphate, pH 7.4, 1 mM PMSF or other protease inhibitors, 1 mM EDTA, and 5% glycerol. For 1 liter, dissolve 6 g sodium phosphate (monobasic), 372 mg EDTA, and 50 mL glycerol in 900 mL deionized water. Use NaOH to adjust pH and bring up the volume to 1 L. Store at 4 °C. Right before use, add the protease inhibitors.
9. Resuspension buffer: 50 mM Tris-HCl (pH 8), 120 mM NaCl, 10% glycerol, 1 mM PMSF. For 1 L, dissolve 6 g Tris-base, 7 g NaCl, 100 mL glycerol in 900 mL deionized water. Use HCl to adjust pH and bring up the volume to 1 L. Store at 4 °C. Right before use, add the protease inhibitors.

## **2.3 For Preparation of Plasma-Enriched Membranes from *P. pastoris* Cells**

To prepare the sucrose gradient: make solutions of 20 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM NaN<sub>3</sub> supplemented with 10, 20, 40 or 60% sucrose. For 100 mL, mix 2 mL of 1 M Tris-HCl solution pH 7.4, 2 mL of 50 mM EDTA solution, 65 mg NaN<sub>3</sub> and 10, 20, 40 or 60 g sucrose, bring up the volume to 100 mL. Store at 4 °C.

## **2.4 For the Purification of the Recombinant Membrane Proteins**

20% n-Dodecyl β-D-maltoside (DDM): dissolve 2 g DDM in 10 mL deionized water. Store at –20 °C.

### **2.4.1 For rCytb<sub>558</sub> (Flag-gp91<sup>phox</sup>/p22<sup>phox</sup>) Purification**

1. Heparin Sepharose 6 Fast Flow column (GE healthcare, France): 20 mL of resin casted into a 20 mL column (20 mm diameter) equilibrated with the purification buffer: 20 mM Tris-HCl, pH 8, 1 mM EDTA, 0.025% DDM.
2. Sephadex 200 column (GE healthcare, France): 20 mL of resin was casted into 20 mL column (10 mm diameter) and equilibrated with the buffer for size exclusion column: 20 mM Tris-HCl, pH 8, 120 mM NaCl, and 0.025% DDM.

#### 2.4.2 For His-gp91<sup>phox</sup> Purification

Buffer A (Ni-NTA equilibrating buffer): 30 mM sodium phosphate (pH 7.5), 0.5 M NaCl, 5 mM Imidazole, and 0.025% DDM.

Buffer B: 30 mM sodium phosphate (pH 7.5), 0.5 M NaCl and applying a linear gradient of imidazole (100 mM).

Buffer C: 20 mM Tris-HCl (pH 7.5), 50 mM NaCl and 0.025% DDM.

#### 2.5 For the Relipidation of the Recombinant Membrane Proteins

1. Phosphate buffer solution (PBS, pH 7.4): dissolve one tablet (Fisher scientific) in 200 mL of deionized water yielding to 0.01 M phosphate buffer, 2.7 mM potassium chloride, and 0.137 M sodium chloride, pH 7.4, at 25 °C.
2. A stock solution of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) from Avanti Polar Lipids at 100 mg/mL.
3. A stock solution of 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (DOPC) from Avanti Polar Lipids at 100 mg/mL.
4. Bio-Beads SM2 (Biorad, Life Science, Marnes-la-Coquette, France).

#### 2.6 For Immunoblotting Test

Blocking buffer: 5% Bovine Serum Albumin in Tris-buffered saline (TBS) solution (50 mM Tris-HCl (pH 7.5) and 150 mM NaCl) containing 0.1% Tween 20.

Anti-His antibody conjugated with horseradish peroxidase (Clontech Inc., France),

rabbit anti-gp91 (54.1) and mouse anti-p22 (FL-195) antibodies (Santa Cruz Biotechnology, Cliniscience, France); anti-rabbit NA934V and anti-mouse NA931 (GE Healthcare, France).

Western Blotting Detection Kit (Amersham Biosciences, France).

#### 2.7 For Cell-Free Assays

1. Activity assay buffer: 20 mM Tris-HCl pH 8.0, 1 mM EDTA, and 120 mM NaCl (*see Note 2*).
2. *Cis*-unsaturated arachidonic acid (C20:4) (AA) 20 mg/mL: dissolve on ice 10 mg of AA in 500 µl absolute ethanol. Aliquot solution and stock at -80 °C (*see Note 3*).
3. NADPH 20 mM: dissolve 4.167 mg NADPH in 250 µl activity assay buffer (*see Note 2*).
4. Equine cytochrome *c* 5 mM: dissolve 18.75 mg Cyt *c* in 300 µl activity assay buffer.
5. Superoxide dismutase (SOD) 2 mg/mL: dissolve 1 mg SOD in 0.5 mL activity assay buffer.

#### 2.8 Equipment

1. Double beam spectrophotometer (e.g., Uvicon XS Secoman).
2. Refrigerated centrifuge and Ultracentrifuge (e.g., Beckman ultracentrifuges).

3. Swinging and fixed angle rotor (e.g., SW 32 Ti and 70 Ti rotors).
4. Orbital shaker incubators.
5. Automatic vortex Bead Beater (e.g., Biosep product) (*see Note 4*).
6. Chromatography system (e.g., Äkta prime system).

### 3 Methods

The classical cell-free assay is a semi-recombinant cell-free assay since the cytosolic proteins used are routinely purified recombinant proteins expressed in *E. coli* while Cyt $b_{558}$  is located in isolated membranes from neutrophils. In the unconventional system developed here, the oxidase membrane proteins are issued from heterologous expression in transgenic yeast. We describe here the preparation of membrane fractions from transgenic yeasts expressing recombinant bovine membrane proteins of the NADPH oxidase. The use of membrane fraction from transgenic yeasts in oxidase cell-free assays is possible since the superoxide anion production is catalyzed only by the heterologous NADPH oxidase proteins, no NADPH-dependent superoxide being detected in membranes from nonexpressing recombinant cyt $b_{558}$  yeast cells.

#### 3.1 Expression of gp91<sup>phox</sup> or rCyt $b_{558}$ (gp91<sup>phox</sup>/p22<sup>phox</sup>) in *Pichia pastoris* Cells

To produce the membrane fraction containing the recombinant heterodimer rCyt $b_{558}$  (gp91<sup>phox</sup>/p22<sup>phox</sup>), *P. pastoris* SMD1168 strain was transformed with the pAO815 (ampicillin resistant) expression vector containing the entire expression cassette with the coding genes for both proteins gp91<sup>phox</sup> (CYBB) and p22<sup>phox</sup> (CYBA), with a fusion tag (FLAG tag) at the N-terminus of gp91<sup>phox</sup>, as described in [3]. To produce the gp91<sup>phox</sup> monomer, *P. pastoris* X33 strain was transformed by electroporation with the pPICZαA (zeocin resistant) expression vector containing the gene coding for the gp91<sup>phox</sup> protein flanked with the Hisx6 fusion tag at the N-terminus [4]. The vector previously linearized is then inserted into the yeast genome by homolog recombination.

#### *Expression procedure:*

1. Spread the recombinant yeast cells on a YPD plate containing 100 µg/mL of zeocin (for X33 strain harboring pPICZα/gp91<sup>phox</sup> vector or 0.05 mg/mL ampicillin (for SMD1168 strain harboring the pAO815/gp91<sup>phox</sup>/p22<sup>phox</sup> vector) and incubate at 30 °C during 3 days.
2. Inoculate BMGY medium supplemented with appropriate antibiotic with transgenic yeast clone and keep culturing overnight at 30 °C and shaking at 225 rpm in incubator shaker.

3. Pellet cells by centrifugation (10 min at  $550 \times g$ ) and resuspend in fresh BMMY medium (with appropriate antibiotic) until reaching  $OD_{600nm}$  of 1.0. Cells are grown in baffled culture flasks (1.5 L) at 30 °C with shaking at 200 rpm.
4. Add 1% methanol (v/v final concentration) every 24 h during 72 h to maintain the expression induction. Harvest cells by centrifugation at  $2500 \times g$  for 15 min and store them at -80 °C until use.

### **3.2 Preparation of the Yeast Total Membrane Fractions (tMF)**

All steps must be done at 4 °C.

1. Thaw and resuspend cells in breaking buffer at  $OD_{600nm} = 50-100$ .
2. Add an equivalent volume of glass beads (0.5 mm diameter) and transfer the mixture into the Bead Beater. Disrupt cells by alternating 30 s vortexing followed by cooling period (1 min 30 s) on ice bath during 20 min.
3. Separate the clear supernatant from the cell debris and glass beads by centrifugation at  $500 \times g$  for 10 min at 4 °C.
4. Collect the total membrane fraction (tMF) by ultracentrifugation at  $130,000 \times g$  for 90 min.
5. Resuspend the pellet (tMF) in a resuspension buffer using potter homogenizer (*see Note 5*).
6. Determine the protein concentration by BCA (bicinchoninic acid assay) with bovine serum albumin as standard (*see Note 6*).

### **3.3 Preparation of the Yeast Subcellular Membrane Fractions**

Although the total membrane fractions obtained as described above are often sufficient, we also may prepare and use subcellular membrane fractions of yeast cells to investigate more precisely the enzyme activity of the oxidase components along the biosynthesis processes.

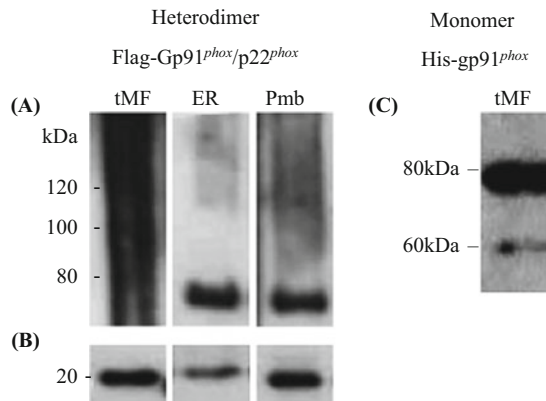
1. In SW32 centrifuge tubes, gently lay 3.7 mL cold 60, 40 then 20% sucrose solutions.
2. Load 0.7 mL of total membrane fractions on the top of the discontinuous sucrose gradient.
3. Leave the centrifuge tubes at 4 °C with minimum disturbance during 4 h to stabilize the gradient.
4. Spin in swinging bucket centrifuge for 17 h (can go longer) at  $110,000 \times g$  in a SW32 Ti rotor at 4 °C.
5. Collect carefully fractions of 700  $\mu$ l from the top to the bottom of the gradient.
6. Analyze the fractions to identify the fractions containing plasma or endoplasmic reticulum membranes with immunoblots using specific antibodies (mouse anti-yeast endoplasmic reticulum (Dpm1p) or anti-ribophorin I and mouse anti-yeast plasma membrane Pma1 anti-bodies).

- For further analysis, pellet the endoplasmic reticulum (ER) and plasma membrane (Pmb) fractions at  $30,000 \times g$  and resuspend in 1 mM EDTA, 10% sucrose or glycerol, 20 mM Tris-HCl pH 8.0 and store at  $-20^{\circ}\text{C}$ .

### 3.4 Immunoblotting Assay

For western blotting assay, usually 1  $\mu\text{g}$  of proteins was used.

- Separate proteins on SDS-PAGE using 10% acrylamide gels.
- Electro-transfer proteins to nitrocellulose membrane (0.2  $\mu\text{m}$ ) at 100 V for 1 h.
- Saturate nonspecific binding with blocking buffer (*see* Sub-heading 2) overnight at  $4^{\circ}\text{C}$ .
- For the detection of the proteins different antibodies can be used. For His-gp91<sup>phox</sup> incubate with anti-His antibody conjugated to horseradish peroxidase diluted at 1:10,000 in TBS buffer. For Flag-gp91<sup>phox</sup>/p22<sup>phox</sup> incubate with the mouse anti-Flag monoclonal antibody (1:3000).
- For specific detection, use rabbit anti-gp91 (54.1) and mouse anti-p22 (FL-195) antibodies at a dilution of 1:1000 and incubate for 2 h at  $4^{\circ}\text{C}$ .
- Use anti-rabbit (NA934V) and anti-mouse (NA931) IgG monoclonal antibodies at a 1:15,000 dilution as secondary antibodies to detect the anti-gp91 and anti-p22 primary antibodies, respectively.
- Use ECL Plus Advance Western Blotting Detection Kit for the revelation of the recombinant gp91<sup>phox</sup> (Fig. 1).



**Fig. 1** Western blots analyses of the different membrane fractions of the heterodimer rCytb<sub>558</sub> and the monomer His-gp91<sup>phox</sup>: (a) analysis with monoclonal anti-Flag; (b) polyclonal anti-p22 of the subcellular membrane fractions containing rCytb<sub>558</sub>. (c) Total membrane fraction (tMF) of monomer His-gp91<sup>phox</sup> analyzed with monoclonal anti-His. tMF: total membrane fraction, ER endoplasmic reticulum and Pmb plasma membrane



### 3.5 Solubilization and Purification of the Recombinant Membrane Proteins

#### 3.5.1 Purification of the Recombinant rCyt<sub>b</sub><sub>558</sub>

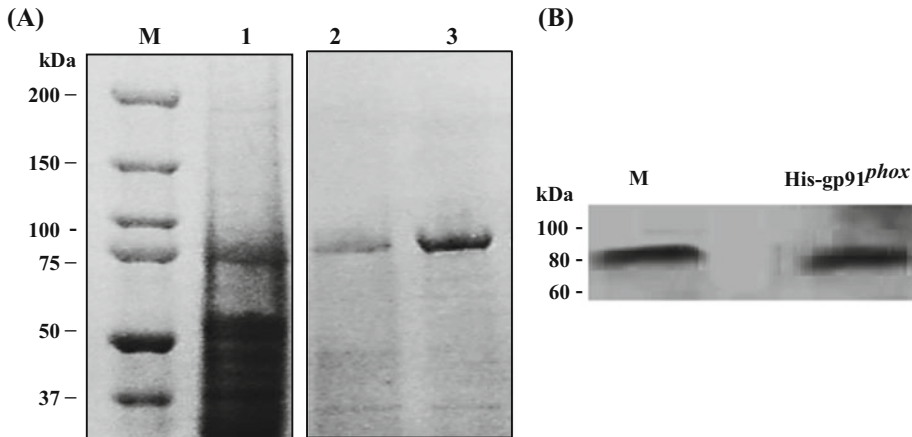
All purification steps were achieved at 4 °C and just before using buffers, add 1 mM PMSF.

1. To solubilize the rCyt<sub>b</sub><sub>558</sub> or gp91<sup>phox</sup> proteins, dilute tFM at 5 mg/mL of total membrane protein in resuspension buffer, add 1.5% DDM (in a detergent/protein ratio of 3:1 (w/w)).
2. Incubate for 1 h at room temperature with gentle agitation.
3. Centrifuge at  $130,000 \times g$  for 90 min at 4 °C. Solubilized membrane proteins are recovered in the supernatant.
4. Load solubilized protein extracts onto Heparine Sepharose column (20 mL) equilibrated with 20 mM Tris-HCl, pH 8, 1 mM EDTA, 0.025% DDM.
5. Wash with 100 mL of the same buffer.
6. Elute rCyt<sub>b</sub><sub>558</sub> with a NaCl gradient (0-1 M NaCl). Usually, rCyt<sub>b</sub><sub>558</sub> is eluted at about 0.4 mM NaCl.
7. Perform spectra of eluted fractions at 280 nm to evaluate the total membrane proteins and difference spectra (400–650 nm) to determine the amount of rCyt<sub>b</sub><sub>558</sub> (*see Note 7*).
8. Pool fractions containing rCyt<sub>b</sub><sub>558</sub> and concentrate using Vivaspin filters (K10).
9. Then to obtain a homogenous sample use a Superdex 200 gel filtration column equilibrated with 20 mM Tris-HCl, pH 8.0, 120 mM NaCl, and 0.025% DDM.

#### 3.5.2 Purification of His-gp91<sup>phox</sup> with Ni-NTA Followed by Gel Filtration Chromatography

The recombinant His-gp91<sup>phox</sup> protein was purified using a column of nickel-nitrilotriacetic acid (Ni-NTA) superflow sepharose followed by gel filtration chromatography using S200 Sephadex resin.

1. Equilibrate the Ni-NTA column using 20 mL of buffer A (*see Subheading 2*).
2. Load sample with a flow rate of 1.5 mL/min.
3. Elute proteins with linear imidazole gradient (5–100 mM) using Buffer B (*see Subheading 2*).
4. Analyze eluted fractions by spectroscopic measurement by performing difference reduced-oxidized spectra to determine the cytochrome content (*see Note 7*).
5. Concentrate fractions and separate proteins on gel filtration chromatography using the buffer C (*see Subheading 2*) with a flow rate of 1.5 mL/min, as described in Subheading 3.5.1.
6. Check the purity of the protein by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 2).



**Fig. 2** Purification and identification of the recombinant gp91<sup>phox</sup> subunit. (a) SDS-PAGE analysis. The total membrane fraction (25 µg; lane 1) was solubilized in 2% DDM and the recombinant gp91<sup>phox</sup> protein was purified on Ni-NTA column (lane 2) followed by gel filtration (15 µg; lane 3). Gels were stained with Coomassie blue. (b) After SDS-PAGE separation of 15 µg of purified protein and membrane transferring, specific identification by the monoclonal anti-gp91 antibody (54.1) from Santa Cruz Biotechnology. M: Magic Mark standard protein (Invitrogen) was used for molecular weight reference

### 3.5.3 Production of Liposomes and Reconstitution of Purified gp91<sup>phox</sup> and rCytb<sub>558</sub> in Liposomes

For the reconstitution of membrane oxidase protein into liposomes, a lipid/protein ratio of 5:1 (w/w) was used.

Vesicles formation was followed by absorbance at 400 nm.

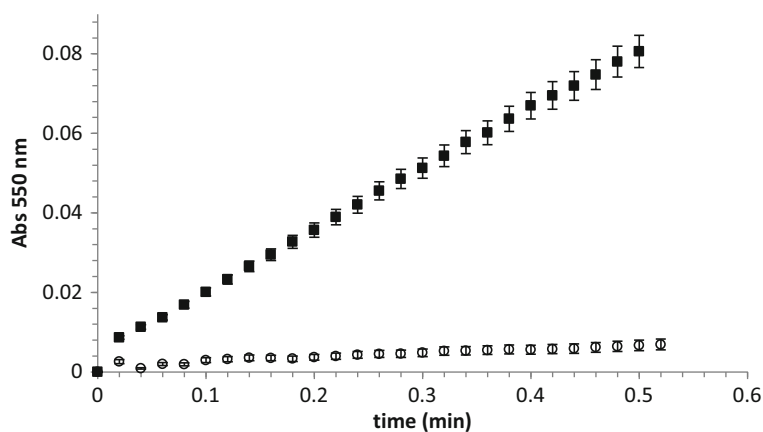
1. Dilute 50 µl of DOPC or DMPC solution (100 mg/mL) four times in PBS.
2. Ultrasonicate lipid by brief pulses until the solution becomes clear (~20 min) preferentially in the presence of nitrogen to avoid lipid peroxydation. The optical density at 400 nm must decrease.
3. Add slowly 200 µl proteins (1 mg/mL). Stir micellar protein-lipid-detergent mixture for 30 min at 30 °C with gentle agitation.
4. Remove DDM by adding 1% Bio-Beads SM2 (1 g of Bio-Beads for 100 mg of DDM) and incubate with gentle agitation for 1 h. Remove Bio-Beads with a brief centrifugation (1 min, 10,200 × g) and place the stock solution containing the proteoliposomes (PL) at -20 °C, until use.

### 3.5.4 The Cell-Free Assays

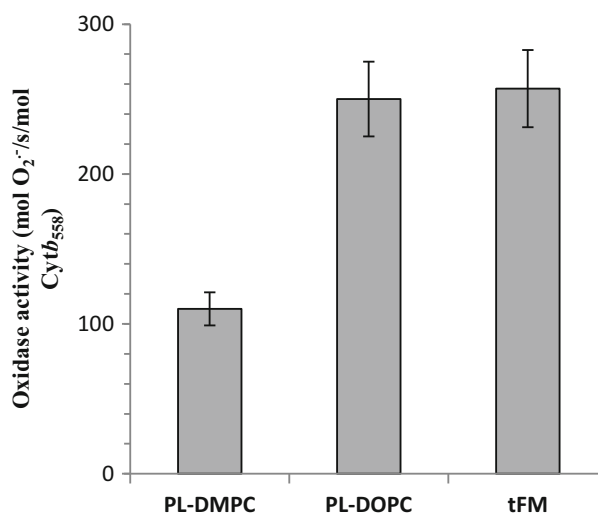
Cell-free assays can be used in very different configurations depending on the components that are mixed together. Here in addition to using a cell-free assay fully constituted with recombinant soluble and membrane proteins, the sophistication is to use also recombinant membrane component reconstituted into artificial phospholipid membranes. The cell-free assays comprise two phases. The first one consists in the oxidase complex assembly insofar as the cytosolic proteins are dispersed in the solution and will have to translocate to the membrane to form the active NADPH oxidase complex.

The second phase is the proper enzyme activity leading to the NADPH-dependent superoxide anion production. Numerous anionic amphiphils act as activators of the NADPH oxidase in cell-free system mimicking the cell signaling processes implying phosphorylation (kinase pathways) and arachidonic release (phospholipaseA2 activation) facilitating protein-protein interactions leading to complex assembly. The canonical arachidonic acid is routinely used in vitro although the effects on the NADPH oxidase components are still unclear (*see Note 8*). The catalytic activity is then visualized by following optically at 550 nm the reduction of ferricytochrome *c* trapping the produced superoxide.

1. Use 1–1.5  $\mu\text{mol}$  of gp91<sup>phox</sup> or rCytb<sub>558</sub> in tFM, ER or Pmb fractions or in PL.
2. Mix with 200 nM of each purified recombinant cytosolic factors (p67<sup>phox</sup>, p47<sup>phox</sup>, and RacQ61L (*see Note 9*)) and arachidonic acid at different concentrations in a total volume reaction of 50  $\mu\text{L}$  activity assay buffer and incubate for 5 min at 25 °C to allow the assembly of the complex.
3. Add 10  $\mu\text{L}$  (5 mM) of ferricytochrome *c* to obtain a final concentration of 100  $\mu\text{M}$ , adjust volume of the reaction to 490  $\mu\text{L}$  with the activity assay buffer.
4. Initiate the reaction with the addition of 10  $\mu\text{L}$  NADPH (20 mM) and rapidly perform measurements of the reduction rate of cytc at 550 nm (OD versus time).
5. Add, on the same reaction mixture, 200 U of superoxide dismutase (SOD) and measure the reduction rate at 550 nm to determine the nonspecific reduction of ferricytochrome *c* (Fig. 3 near here).



**Fig. 3** SOD-sensitive NADPH-oxidase activity of total membrane fractions containing rCytb<sub>558</sub>. Measure in the absence (*black square*) or in the presence (*empty circle*) of 50  $\mu\text{g/mL}$  SOD. NADPH oxidase activity assay was done at 25 °C with optimal arachidonic acid concentration



**Fig. 4** The “artificial cell-free assay.” Anion superoxide production was measured with total membrane fraction replaced with PL-DMPC or PL-DOPC

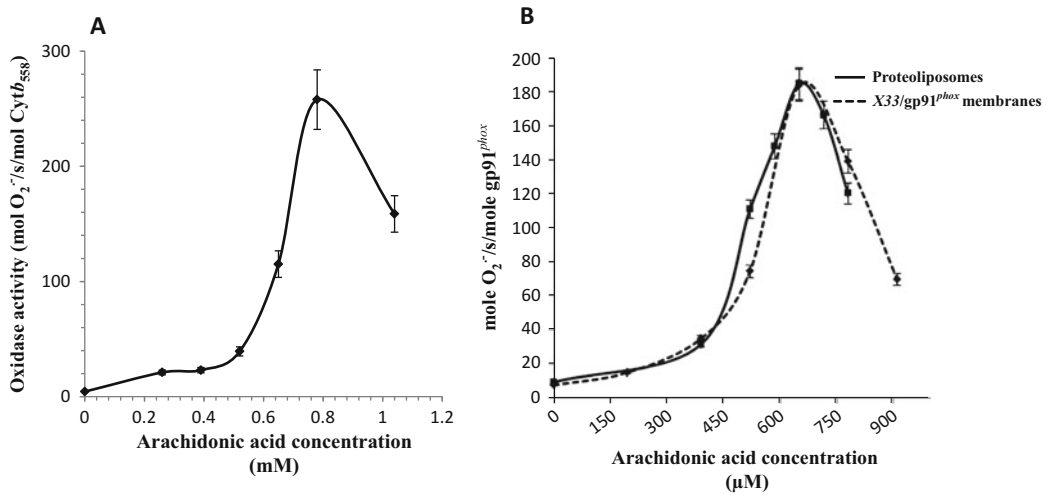
6. Calculate the amount of superoxide generated using the molar absorption coefficient of  $21.1 \text{ mM}^{-1} \text{ cm}^{-1}$  and normalize to 1 pmole of gp91<sup>phox</sup> used into the sample.
7. The rate of superoxide production can be compared for different experimental conditions in particular regarding the membrane environment such as different membrane lipid composition (Fig. 4).

### 3.5.5 NADPH Oxidase Activation Specificities

Arachidonic Acid  
Activation: Dose-Response  
Curve

The final concentration of AA causing maximal activation has to be optimized. Depending on the cell-free system considered, this optimal value is highly variable (even within the same species it can be different as we often observed from human membrane fractions depending on the blood donor). The differences are correlated to species variation (human, bovine, etc.) but the fundamental reasons lie behind important parameters such as different ratio of membrane protein/lipids and off course protein and lipid composition (*see Note 10*). The concentration-response curve that plots the enzyme activity vs the AA concentration has commonly a bell shape. Such a curve is a characteristic of phagocyte NADPH-oxidase activation, indicating that the activation process through the activator molecule is similar to native enzyme.

- Realize cell-free assays as described in Subheading 3.6 with membrane fractions (tMF, PL, etc.) with increasing concentration of AA to plot AA-dependent bell-shape activation curve: start measure in the absence of AA up to 1 mM. (Fig. 5a, b).



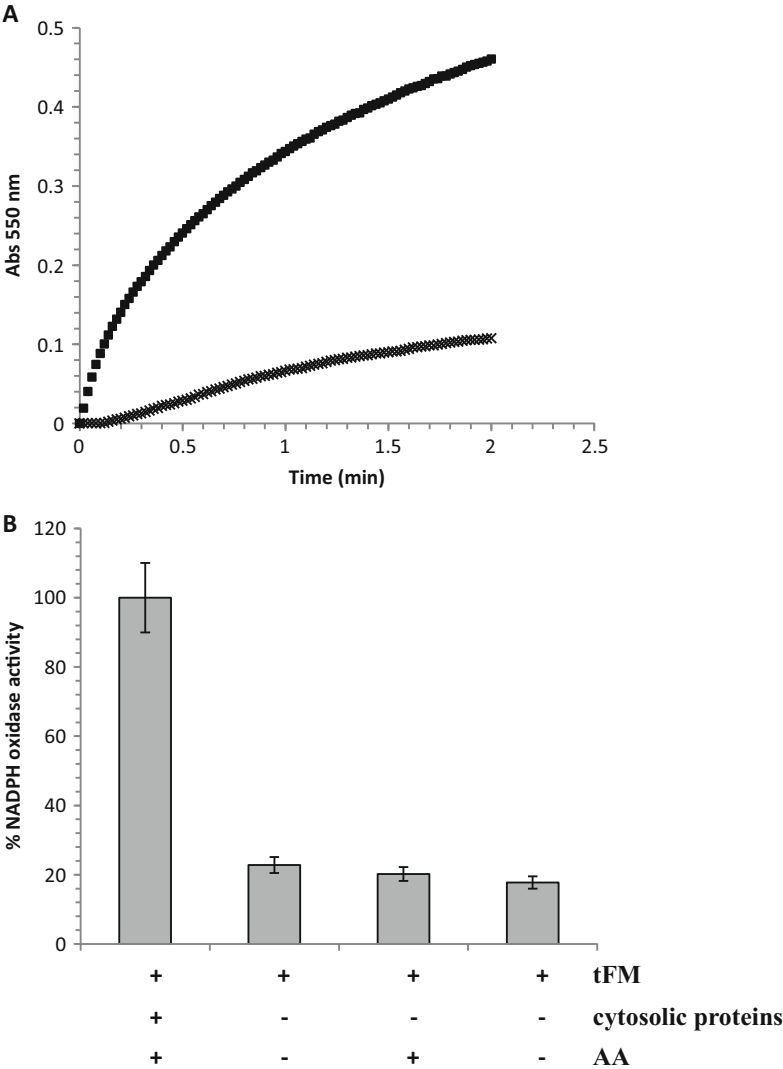
**Fig. 5** Activation of the NADPH-oxidase complex by arachidonic acid. **(a)** Activity assays were performed in cell-free system using total membrane fraction of rCytb<sub>558</sub> with different concentrations of arachidonic acid at 25 °C. **(b)** NADPH oxidase activity of the reconstituted gp91<sup>phox</sup> in DOPC liposomes (~2.24 nM of gp91<sup>phox</sup>) and of gp91<sup>phox</sup> total membrane fraction. Measurements of the superoxide production by PLs were performed with the standard cyt c reduction assay in the absence of any cytosolic proteins and increasing concentrations of arachidonic acid. Results are presented as the mean ± SD ( $n = 3$ )

Cytosolic Protein  
Dependences of the NADPH  
Oxidase Activation

Performing the same experiment as in Subheading 3.5.4. (Fig. 3) in the absence or in the presence of cytosolic proteins or AA is an interesting control of activation stage. The implication of each component in the oxidase activity of rCytb<sub>558</sub> or gp91<sup>phox</sup> alone can be investigated using different sets of experimental condition of the cell-free assays. The absence of one of the components impedes efficient superoxide production.

1. To show the importance of incubation time for complex assembly, mix membrane fractions without or with cytosolic proteins.
2. Then add AA, cytc and NADPH, complete to 500 μl with buffer, and measure the production of superoxide anions by following absorption at 550 nm (Fig. 6a) (see Note 11).

The different oxidase activities of total membrane fraction containing rCytb<sub>558</sub> can be measured in infinite experimental conditions. Here are compared the oxidase activities with or without cytosolic proteins and with and without AA (Fig. 6b).



**Fig. 6 (a)** Importance of complex assembly to produce anion superoxide in cell-free assay. NADPH oxidase activity was followed with (*dark square*) or without pre-incubation (*cross*) of rCytb<sub>558</sub> with cytosolic proteins and in the presence of optimal concentration of AA. **(b)** rCytb<sub>558</sub> activation with cytosolic protein and with arachidonic acid. Cell-free assays were done in the absence or presence of AA or cytosolic protein or both. NADPH oxidase measured in the presence of optimal concentration of AA and cytosolic protein is considered 100%. The value of this reference activity is 350 mol O<sub>2</sub><sup>•-</sup>/s/mol rCytb<sub>558</sub>

#### 4 Notes

1. The measurement of the generated superoxide anions followed by cytochrome c reduction is a classical technique that can be employed to examine the catalytic parameters of all enzymes implicated into oxygenated redox reactions.

2. It is best to prepare this fresh just before use. Alternatively, it can be dissolved in water for better stability.
3. Small aliquots volumes must be done to avoid oxidation of arachidonic acid.
4. For reproducibility, we modified the apparatus in order to monitor automatically the cycle of vortex/pause periods.
5. If tFM is used for the separation in sucrose gradient, solubilize in 10% sucrose solution.
6. About 6 mg proteins/L of yeast culture was usually obtained.
7. The amount of gp91<sup>phox</sup> or gp91<sup>phox</sup>/p22<sup>phox</sup> is spectrophotometrically determined from the difference absorption spectra of the redox protein in its reduced minus oxidized form. The reduced form is obtained by adding few grain of Na dithionite. The Cyt<sub>b</sub><sub>558</sub> heme amount is quantified by measuring the absorbance difference between the peak at 427 nm and the hollow at 411 nm using a millimolar extinction coefficient of  $\Delta\epsilon_{427-411} = 200 \text{ mM}^{-1} \text{ cm}^{-1}$  [7].
8. The NADPH oxidase complex can also be directly and efficiently activated by other amphiphilic molecules, such as sodium dodecyl sulfate (SDS). Arachidonic acid (AA) is a well-known second messenger in signaling pathways which potentiate the NADPH oxidase complex in vitro. Direct impact of AA on gp91<sup>phox</sup> leads to conformational changes, which might participate in the NADPH oxidase activation [8, 9]. AA interacts also directly with the cytosolic proteins [10, 11] with recently new insights of the role of *cis*-AA into the interaction of p67<sup>phox</sup>-Rac with gp91<sup>phox</sup> [12]. It was proposed that anionic amphiphils perturb the intramolecular bonds in p47<sup>phox</sup> between the polybasic domain and the SH3 tandem mimicking the phosphorylation events [13].
9. Purification protocols of the cytosolic proteins used are described in [2]. In the cell-free assays, the mutant of the Rac1 protein (Rac1Q61L) which is in its active GTP bound form is often used. It presents the advantage that GTP is not necessary to be added to obtained the active Rac form.
10. The partitioning of the amphiphilic molecules depends on the membranes composition and AA will have a more diluted effect in membranes containing less target proteins (cyt<sub>b</sub><sub>558</sub>). In the literature, depending on the human semi-recombinant cell-free system considered, the optimal AA concentration values range from 30  $\mu\text{M}$  [11, 14, 15], to 200  $\mu\text{M}$  [16]. With the bovine cell-free systems, the maximal O<sub>2</sub><sup>•-</sup> production occurred at 150–200  $\mu\text{M}$  of AA, with neutrophil bovine membrane fractions (containing in general >0.06 mg of Cyt<sub>b</sub><sub>558</sub>/mg of total membrane proteins), while this value reaches 750–800  $\mu\text{M}$

with yeast membrane fraction containing the bovine recombinant proteins ( $< 0.01$  mg of Cyt $b_{558}$ /mg of total membrane proteins). A more diluted target (Cyt $b_{558}$ ) protein will need higher AA concentration for activation. However, the AA interactions with the NADPH oxidase components and cellular membrane are still open questions that need to be investigated more.

11. The purified gp91<sup>phox</sup> or gp91<sup>phox</sup>/p22<sup>phox</sup> in detergent showed almost no activity. When reconstituted into PL, gp91<sup>phox</sup>/p22<sup>phox</sup> still need to be activated by the cytosolic proteins while in the absence of p22<sup>phox</sup>, gp91<sup>phox</sup> produce superoxide in the absence of cytosolic subunits but remain AA-dependent.

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## References

1. Dagher MC, Pick E (2007) Opening the black box: lessons from cell-free systems on the phagocyte NADPH-oxidase. *Biochimie* 89:1123–1132
2. Baciou L, Erard M, Dagher MC, Bizouarn T (2009) The cytosolic subunit p67<sup>phox</sup> of the NADPH-oxidase complex does not bind NADPH. *FEBS Lett* 583:3225–3229
3. Ostuni MA, Lamanuzzi LB, Bizouarn T, Dagher MC, Baciou L (2010) Expression of functional mammal flavocytochrome b(558) in yeast: comparison with improved insect cell system. *Biochim Biophys Acta* 1798:1179–1188
4. Ezzine A, Souabni H, Bizouarn T, Baciou L (2014) Recombinant form of mammalian gp91(phox) is active in the absence of p220(phox). *Biochem J* 462:337–345
5. Molshanski-Mor S, Mizrahi A, Ugolev Y, Dahan I, Berdichevsky Y, Pick E (2007) Cell-free assays: the reductionist approach to the study of NADPH oxidase assembly, or “all you wanted to know about cell-free assays but did not dare to ask”. *Methods Mol Biol* 412:385–428. Humana Press, Totowa, NJ
6. Pick E (2014) Cell-free NADPH oxidase activation assays: “In Vitro Veritas”. *Methods Mol Biol* 1124:339–403. Humana Press
7. Light DR, Walsh C, O’Callaghan A, Goetzl E, Tauber A (1981) Characteristics of the cofactor requirements for the superoxide-generating NADPH oxidase of human polymorphonuclear leukocytes. *Biochemistry* 17:1468–1476
8. Doussiere J, Gaillard J, Vignais PV (1996) Electron transfer across the O-2(−) generating flavocytochrome b of neutrophils. Evidence for a transition from a low-spin state to a high-spin state of the heme iron component. *Biochemistry* 35:13400–13410
9. Souabni H, Thoma V, Bizouarn T, Chatgililoglu C, Siafaka-Kapadai A, Baciou L, Ferreri C, Houee-Levin C, Ostuni MA (2012) Trans Arachidonic acid isomers inhibit NADPH-oxidase activity by direct interaction with enzyme components. *BBA-Biomembranes* 1818:2314–2324
10. Shiose A, Sumimoto H (2000) Arachidonic acid and phosphorylation synergistically induce a conformational change of p47(phox) to activate the phagocyte NADPH oxidase. *J Biol Chem* 275:13793–13801
11. Swain SD, Helgersson SL, Davis AR, Nelson LK, Quinn MT (1997) Analysis of activation-induced conformational changes in p47(phox) using tryptophan fluorescence spectroscopy. *J Biol Chem* 272:29502–29510



12. Matono R, Miyano K, Kiyohara T, Sumimoto H (2014) Arachidonic acid induces direct interaction of the p67(phox)-Rac complex with the phagocyte oxidase Nox2, leading to superoxide production. *J Biol Chem* 289:24874–24884
13. Groemping Y, Lapouge K, Smerdon SJ, Rittinger K (2003) Molecular basis of phosphorylation-induced activation of the NADPH oxidase. *Cell* 113:343–355
14. Curnutte JT (1985) Activation of human neutrophil nicotinamide adenine-dinucleotide phosphate, reduced (triphosphopyridine nucleotide, reduced) oxidase by arachidonic-acid in a cell-free system. *J Clin Invest* 75:1740–1743
15. Clark RA, Leidal KG, Pearson DW, Nauseef WM (1987) NADPH oxidase of human neutrophils - subcellular-localization and characterization of an arachidonate-activatable superoxide-generating system. *J Biol Chem* 262:4065–4074
16. Ligeti E, Pizon V, Wittinghofer A, Gierschik P, Jakobs KH (1993) GTPase activity of small GTP-binding proteins in HL-60 membranes is stimulated by Arachidonic-acid. *Eur J Biochem* 216:813–820

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